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BIOCHEMICAL AND SEROLOGICAL CHARACTERISATION OF A CALF ROTAVIRUS

by

Helen Killen

A thesis presented for the degree of

Doctor of Philosophy

to

The University of Warwick

DECLARATION

This thesis is submitted by the undersigned to the University of Warwick for examination for the degree of Doctor of Philosophy. The work herein is entirely my own and has not been submitted as an exercise for a degree to this or any other University.

Helen Killen

Helen Killen

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Helen Killen

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Part of this thesis is included in the following published paper:

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Identification of a neutralization-specific
antigen of a calf rotavirus.

Journal of General Virology 62, 297-311.

To my parents.

Abbreviations

α	anti.
AMD	actinomycin D
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
BME	β -mercaptoethanol
BSA	bovine serum albumin
C'	complement
CF	complement fixation
CIEP	counter immunoelectrophoresis
Con A	Concanavalin A
CsCl	caesium chloride
CPV	cytoplasmic polyhedrosis virus
CTL	cytotoxic T lymphocyte
DEAE-cellulose	diethylaminoethyl cellulose
DMSO	dimethyl sulphoxide
DOC	sodium deoxycholate
ds	double stranded
<u>E.coli</u>	<u>Escherichia coli</u>
EDIM	epizootic diarrhoea of infant mice
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GMEM	Glasgow's minimal essential medium
HA	haemagglutinin
HI	haemagglutination inhibition
IAHA	immune adherence haemagglutination assay
IEM	immune electron microscopy
IF	immunofluorescence
Ig	immunoglobulin

Abbreviations (Cont'd.)

In	infected cells
IP	immune precipitation
moi	multiplicity of infection
NP ₄₀	Nonidet P40
NTP	nucleoside triphosphate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEP	phosphoenol pyruvate
p.f.u.	plaque forming units
p.i.	post infection
poly-A	poly-adenosine
PP0	2,5,-diphenyl oxazole
RNAase	ribonuclease
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
r.p.m.	revolutions per minute
S	Svedberg constant of sedimentation coefficient
<u>S.aureus</u>	<u>Staphylococcus aureus</u>
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
SVP	sub viral particle
TCA	trichloroacetic acid
TEMED	N,N,N'-N'-tetramethylethylene diamine
Triton X-100	150-octylphenoxypolyethoxyethanol
Tris	tris (hydroxymethyl) methylamine
t.s.	temperature sensitive
Un	uninfected cells
VP	virus protein
	(All nucleotides and nucleosides are written in their usual abbreviated form, e.g. adenosine-5'-triphosphate)
ATP	

SUMMARY

mRNA was transcribed in vitro by the endogenous RNA transcriptase of calf rotavirus. This mRNA had a mean sedimentation coefficient of 12S, and hybridised to genomic double stranded RNA. Rotavirus mRNA appeared not to be polyadenylated as less than 1% of it was retained by oligo dT cellulose. Methyl groups from S-adenosyl methionine were incorporated in this system into RNA which co-sedimented with ³²P-labelled mRNA on sucrose gradients, and the methyl groups were shown to be present in the form of an alkali-resistant cap which co-migrated with the reovirus 5' cap on DEAE cellulose with a net negative charge of -5 to -6.

In vitro translation of the in vitro transcription products gave 12 polypeptides, 11 of which had the same mobility on PAGE as virus-specific polypeptides seen in infected cells treated with tunicamycin (i.e. in which glycosylation was inhibited). These were VP1, VP2, VP3, VP4, VP5, VP6, VP8, VP9, vpr7, VP11 and VP12. The VP2, VP3, VP5, VP6, VP8, VP9 and VP12 from in vitro translation and from infected cells were compared by peptide mapping and were shown to be the same proteins. Two of the primary gene products (VP12 and vpr7) become glycosylated in infected cells to VP10 and VP7 respectively, and these are cleaved to VP10c, VP7.1 and VP7.2. Virus structural proteins which are also seen in

infected cells are VP1, VP2, VP6, VP7.1, VP7.2 and VP10c. Virus particles also have polypeptides VP3*, VP4*, VP4.2, VP5* and VP4.3 which are not found in infected cells, and are all produced during trypsin activation of virus particles. VP4.2 and VP4.3 are produced by cleavage of VP3 by trypsin, and VP3*, VP4* and VP5* are produced by cleavage of some of VP2. Thus, only three primary gene products (VP1, VP2 and VP6) are seen in virus particles while the other eight structural proteins (VP3*, VP4*, VP4.2, VP5*, VP7.1, VP7.2, VP10c and VP4.3) are the result of post-translational modification of primary gene products.

Immune precipitation of polypeptides from infected cells revealed that VP2, VP3*, VP4*, VP6, VP7/7.1 and VP8 were efficiently precipitated, VP10/10c were inefficiently precipitated, and VP1 and VP12 were not precipitated by anti-calf rotavirus serum. Immune precipitation using heterologous antisera led us to conclude that VP2, VP6 and VP8 are group-specific antigens, and VP3, VP4.2 and VP7/7.1 behave as type-specific antigens.

Monospecific polyclonal antisera were raised in guinea-pigs against the purified calf rotavirus polypeptides VP1, VP2, VP3*, VP4*, VP4.2, VP6, VP7.1, VP7.2 and VP10. All of the antisera gave a similar pattern of cytoplasmic immunofluorescence in rotavirus-infected cells, and spots of fluorescence of varying intensity with different antisera were also seen over the nucleus. Immune precipitation showed that VP2 was precipitated

by antiserum to VP2 (α -VP2) and α VP3*,4* and VP6 by α VP6, α VP7.1 and α VP7.2 both precipitated the same range of proteins from infected cells (VP7, VP7.1 and VP7.2) or from virions (VP7.1 and VP7.2) VP10, either from virions or infected cells, was not precipitated by α VP10. The only antiserum which efficiently neutralized infectivity was α VP7.2. There were low levels of neutralization with α VP10 (but the results varied from experiment to experiment), and trace levels with α VP6. Neutralization by α VP7.2 and α VP10 was enhanced by complement. α VP10 and α VP6 both appeared to block neutralization by α VP7.2. α VP7.1 and the other antisera did not neutralize even though α VP7.1 agglutinated double shelled particles as seen in immune electron microscopy to a greater extent than α VP7.2. Both VP7.1 and VP7.2 were shown to be glycoproteins by tunicamycin treatment of infected cells. Core particles only were agglutinated by α VP10. All the evidence leads to conclude that there are major neutralizing antigenic determinants present on VP7.2, a minor component of the outer shell of the virion.

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INTRODUCTION

CHAPTER 1

INTRODUCTION

Bishop et al. and Flewett et al. identified the rotaviruses as agents of human infantile gastroenteritis in 1973. All the previously identified viruses of the reovirus family had been thought of as 'orphan' viruses as they were relatively non-pathogenic in humans. Preliminary surveys have since shown rotaviruses to be the most important pathogen in gastroenteritis of younger children (Kapikian et al., 1980), and this disease is one of the leading causes of sickness and childhood mortality in developing countries, as it is estimated to cause 5-18 million deaths annually (Kapikian et al., 1980). The rotaviruses are also prominent in gastrointestinal diseases of young farm animals (Flewett and Woode, 1978; McNulty, 1978; Woode 1979), so this new group poses a problem of both economic and medical importance.

The reoviruses have always been the subject of much interest because of their many unusual biological properties such as their double protein capsid structure, ds RNA genome and conservative method of replication. Also, because of their ease of cultivation, relative simplicity and their ability to transcribe enormous quantities of mRNA, they have been found to be a useful model for many aspects of eucaryotic cell biology. The wealth of information available about reovirus has been of enormous importance in rotavirus research. This chapter reviews the current knowledge about rotaviruses with particular reference to reovirus,

the prototype of the family, which has been a model for the study of rotaviruses and which has provided much of the information and methodology of rotavirus molecular biology.

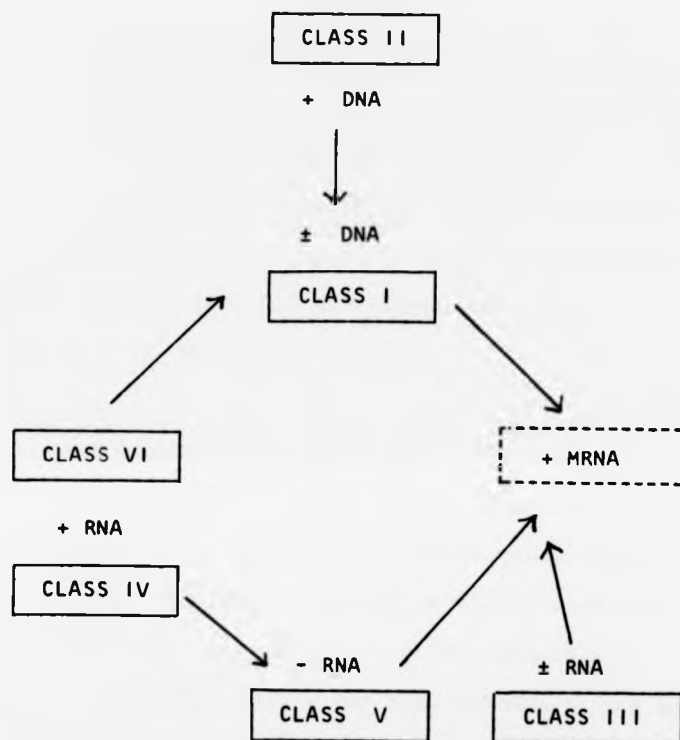
1.1 The Reovirus Family - History and Classification

Reoviruses were first discovered in the early 1950's during intense research into enteric viruses in an attempt to study acute poliomyelitis and to develop poliovirus vaccines. New viruses were isolated from the human alimentary tract and were termed ECHO (enteric cytopathic human orphan) viruses. Most of these were identified as enteroviruses belonging to what was later known as the Picornavirus family, but one of the ECHO groups known as ECHO 10, which were larger than other ECHO viruses, were subsequently classified as reovirus serotypes 1, 2 and 3 (Ramos-Alvarez and Sabin, 1954). Further isolates were derived from macaca monkeys (Rosen, 1960) and a human isolate in the brains of suckling mice (Stanley, 1961). Since then, hundreds of isolates have been found with morphological similarities from respiratory or alimentary tracts, but not associated with any particular disease - hence the acronym (REO = respiratory enteric orphan).

The discovery of dsRNA in reoviruses (Gomatos et al., 1962; Gomatos and Tamm, 1963) subsequently provided the strongest distinguishing characteristic of the family, and all reovirus-like isolates with dsRNA were found to have segmented genomes. The reovirus family belong to class III of the Baltimore classification scheme outlined in Fig.1 (Baltimore, 1971), and reoviruses were the source of the first dsRNA to be found in nature. Their 'conservative'

FIG. 1 THE BALTIMORE CLASSIFICATION SCHEME FOR VIRUSES

(Baltimore, 1971).



NOTE: Autonomous parvoviruses have DNA⁺.

method of multiplication (i.e. mRNA is transcribed only from a single strand of the genome) was another unusual genetic feature of this unique group of viruses.

Joklik (1974) defined two criteria necessary for classification of new isolates into the reovirus family. Firstly, the viruses must possess quasispherical capsids 60-80 nm in diameter and display icosahedral symmetry, and secondly their genomes should consist of 10-12 molecules of double stranded RNA with a combined molecular weight of about 15×10^6 daltons.

Viruses in this family have been found which infect plants, arthropods and vertebrates. These have now been divided into five named genera - by the International Committee on Taxonomy of Viruses in 1978 - these are reovirus, orbivirus, rotavirus, phytoreovirus and fijivirus. Cytoplasmic polyhedrosis virus also belongs in the same family but has not been assigned a genus name. Fig.2 outlines the properties and divisions of the reovirus family, described below:

1. The reovirus genus contains three mammalian serotypes and 5 avian serotypes. These viruses infect their host directly without a vector.
2. The orbiviruses have a vertebrate host as well as an insect vector. This is probably the largest genus in the reovirus family with 10 groups, many

FIG.2

OUTLINE OF THE REOVIRUS FAMILY

(Reproduced from Stanley, 1981)

PROPERTIES OF WELL DEFINED MEMBERS

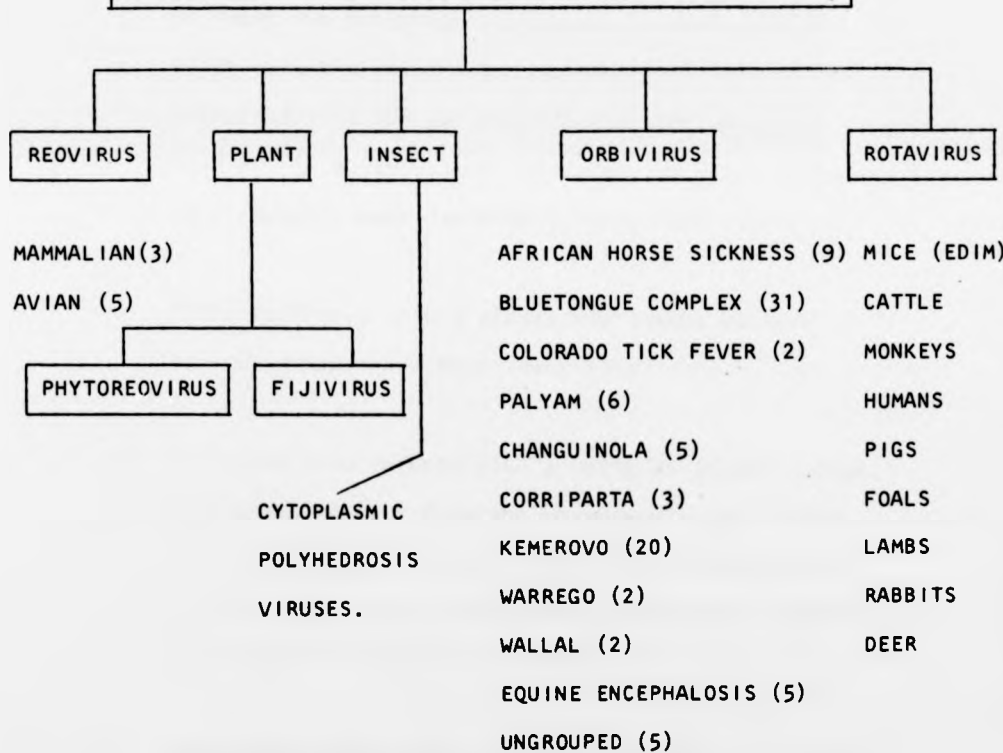
CAPSID OF 60-80 nm DIAMETER, ICOSAHERAL SYMMETRY
(WITH INNER AND OUTER CAPSID).

GENOMES OF 10-12 MOLECULES OF DOUBLE STRANDED RNA

VIRION CONTAINS TRANSCRIPTASE.

CYTOPLASMIC REPLICATION.

RESISTANCE TO LIPID SOLVENTS.



2. (Cont'd.)
of which have several serotypes. Many of the orbiviruses are still ungrouped.
3. The rotaviruses infect many mammals and birds without a vector. The number of serotypes has not been established but there is at least one serotype for each animal host and three serotypes of human rotaviruses have been identified. There are probably at least two sub-groups of rotavirus, since viruses which resemble rotaviruses morphologically and have a rotavirus-like RNA pattern but are serologically distinct (i.e. they lack the rotavirus group antigen(s)) have recently been isolated (see section 1.2.6).
4. Phytoreoviruses infect plants via insect vectors. Several groups have been identified.
5. Fijivirus also infects plants using an insect vector. This genus differs from the phytoreoviruses in the structure of their protein shell (fijiviruses have spikes), the type of vector and their genome pattern (10 segments in comparison to 12).
6. Cytoplasmic polyhedrosis viruses, although obviously in the reovirus family, have not been grouped into any genus. These viruses infect at least 80 species of

6. (Cont'd.)

insect but have no other host. This group contains several serologically distinct viruses.

The above genera have no serological relationship with each other, and each genus also has a characteristic RNA pattern which has been found useful in preliminary characterisation. Initial characterisation, however, is still based on morphology and structure. Table 1 illustrates some of the common features and differences in the structure of rotaviruses, reovirus and orbiviruses.

The mammalian reoviruses are characteristically non-pathogenic although reovirus serotypes 1 and 3 cause a variety of symptoms in newborn rodents (Walters et al., 1963; 1965; Stanley and Walters, 1966) which are discussed in Section 1.3.9. The plant reoviruses are pathogenic, causing galls (fiji disease virus), stunting (rice dwarf virus), dwarfing (maize rough dwarf virus) and tumours (wound tumour virus) (Joklik, 1974), and orbiviruses also cause a variety of quite severe diseases in mammals - bluetongue virus causes cyanosis of the mouth and fever in sheep, and possible oedema of head and neck, African Horse Sickness virus causes fever and pulmonary oedema in horses and Colorado Tick fever causes mild febrile illness in man (Stanley, 1981). The rotaviruses are considered to be the most serious pathogens as they cause acute gastroenteritis in animals and humans very readily, are associated with up to

18 million deaths of children alone per year [Kapikian et al. (1980)], and can cause up to 80% mortality in farm animals. The next section describes the rotaviruses and their associated clinical features.

CHARACTERISTICS OF ANALOGOUS LAYERS OF VIRUS PARTICLES REPRESENTATIVE OF THREE GENERA OF THE REOVIRIDAE FAMILY

T A B L E 1 (From Stanley, 1981)

GENUS	VIRUS PARTICLE LAYERS		
	OUTER	MIDDLE	INNER
ROTAVIRUS	Sharply defined margin forming a continuous covering over the main capsid	Well-defined capsomers arrayed with $T = 3$ primary symmetry and $T = 9$ secondary symmetry	Particles without resolved subunits but with 5-3-2 symmetry characteristic of an icosahedron.
REOVIRUS	Featureless layer located either over or among capsomers of the main capsid.	Capsomers difficult to define; $T = 3$ symmetry resolved only with rotational image enhancement.	<ol style="list-style-type: none"> 1. Particles with subunits considered to be capsomers. 2. Particles with capsomers and projections at vertices of an icosahedron.
ORBIVIRUS (bluetongue serotype 10)	Fuzzy, indistinct layer covering the main capsid.	Well-defined capsomers arrayed with $T=3$ symmetry, with secondary symmetry similar to that of rotavirus.	Particles without resolved subunits, but with 5-3-2 symmetry characteristic of an icosahedron.

1.2 The Rotaviruses

1.2.1 Discovery and preliminary characterisation

Virologists had long suspected that most outbreaks of gastroenteritis were caused by viruses, since infections attributable to Salmonella spp., Shigella spp. and Escherichia coli comprised only a small percentage of the total number of acute cases. However, demonstration of these viruses proved difficult until development of the use of specific pathogen free animals for experimental purposes and of direct electron microscopy of crude samples. The former overcame problems, derived from other causes of diarrhoea, frequent spontaneous diarrhoea in control animals and from previous immunity of the animals, which had confused the situation. Rotaviruses, coronaviruses, parvoviruses, adenoviruses, astroviruses and calici-like viruses have all been shown to be significantly associated with gastroenteritis in young and newborn animals (Woode, 1979). Rotaviruses are important agents of gastroenteritis in animals (Kurstak et al., 1981) and are the major cause of diarrhoea in children (DuPont et al., 1977; Middleton, 1977).

The first rotavirus isolated from animals with gastroenteritis was the epizootic diarrhoea of infant mice (EDIM) virus. This virus was 65-76 nm in diameter, was very infectious, caused diarrhoea in suckling mice

1.2.1. (Cont'd).

and was resistant to heat and ether (Kraft, 1957; 1966; Adams and Kraft, 1963). It was also stable to storage and acid treatment, contained RNA (Much and Zajac, 1972) and was cultivatable in organ cultures of intestinal epithelium but not in cell culture (Rubinstein et al., 1971). Electron microscopic studies of sections of infected mouse gut by Adams and Kraft (1967) and Banfield et al. (1968) found that the virus replication was limited to cells at tips and sides of villi while crypt cells were not infected. The epithelial brush border was normal in early stages of infection but later became short, thick and irregular; and infected cells were shed into the intestinal lumen releasing large numbers of virus particles. The virus replication sites were entirely cytoplasmic, generally in areas of 'viroplasm' and in cisternae of rough endoplasmic reticulum (see Section 1.3.8). Many of the virus particles were surrounded by membrane and elongated and tubular forms of the virus were also found (Banfield et al., 1968). The pattern of multiplication observed was most similar to that of the reoviruses.

The simian rotavirus (SA-11) and the 'O' agent were isolated from a vervet monkey and from pooled intestinal washings of cattle and sheep respectively and were cultivatable in primary vervet monkey kidney cells

1.2.1. (Cont'd).

(Malherbe and Strickland-Cholmley, 1967). The viruses were morphologically identical and thought to resemble orbiviruses with a sharply defined outer capsid surrounding an inner capsid, and the virus was frequently enveloped. Both were pH 4 stable, and SA-11 was also resistant to ether and chloroform (Lecatsas, 1972; Els and Lecatsas, 1972).

The real interest in rotaviruses began when Mebus et al. (1969) succeeded in transmitting gastroenteritis to gnotobiotic calves by inoculating them intraduodenally with bacterial-free filtrates of faeces from calves with diarrhoea, and later by feeding the calves with virus. The animals were found to develop diarrhoea within 24-48 hours, and large numbers of 65 nm virus particles were found in the faeces. Faecal cells were stainable by immunofluorescence using antiserum raised in rabbits from the purified virus. Reovirus-like viruses were isolated and eventually adapted to tissue culture, although this virus did not grow readily like SA-11 and required several passages in primary foetal bovine kidney cells (Mebus et al., 1971).

The first human rotavirus was discovered in 1973. Bishop et al. (1973) carried out electron microscopy on

1.2.1 (Cont'd).

thin sections of duodenal biopsies from young children with acute gastroenteritis. These sections showed dilated cytoplasmic vesicles containing large numbers of virus particles, some of which appeared to be budding into these vesicles from areas of 'viroplasm'. Bishop et al. called these particles 'orbiviruses'. Flewett et al. (1973) identified reovirus-like particles in stool suspensions of young children using electron microscopy. The viruses had a double layered capsid and a well defined rim. Because of their wheel-like morphology, the name 'rotavirus' was devised and proposed by Flewett. Bishop et al. (1974) confirmed the presence of virus in faecal samples, and Middleton et al. (1974) found viruses in duodenal biopsies and faeces of infected children and showed that intestinal cells could be stained by indirect immunofluorescence with anti-rotavirus serum. The presence of rotavirus antibody was confirmed in the serum of all but one normal adult volunteer, who was successfully infected intra-duodenally with a faecal filtrate and developed rotaviral infection. Kapikian et al. (1974) demonstrated virus-like particles in faeces and elevated antibody levels during the disease, and noted a morphological and antigenic similarity between the human rotavirus and the mouse and calf isolates. Human rotaviruses have now been associated with a large proportion of cases of gastroenteritis in young children, the incidence being highest in children

1.2.1 (Cont'd.)

from 1-4 years of age, and decreasing thereafter (Flewett and Woode, 1978). In less developed countries, the disease is a severe problem and thought to be responsible for high mortality, although more in depth etiological studies are needed. (Kapikian et al., 1980; Greenberg et al., 1981).

Rotaviruses have now been found in a wide variety of mammals and birds (McNulty, 1978; McNulty, 1979). They are all morphologically similar and have common antigenic properties (Woode et al., 1976), but at least one completely new antigenic group, which is unreactive with normal rotavirus antisera by most serological tests, has now been identified (Saif et al., 1980; McNulty et al., 1981; Bridger et al., 1982).

1.2.2 Classification

The association of rotaviruses with diarrhoea of young farm animals and of children led to a comparative study of the group. They were originally termed 'reovirus', 'reovirus-like' or 'orbivirus' due to their similarities to these organisms, but on further investigation significant morphological differences were noted between the rotaviruses and other members of the reovirus family. Analysis of rotaviral RNA showed it to be double stranded and segmented like that of reoviruses, but to consist of 11 segments with

1.2.2. (Cont'd).

a characteristic basic pattern common only to the rotaviruses (Newman et al., 1975; Rodger et al., 1975). The combined molecular weight of RNA was about 11×10^6 daltons (McNulty, 1979). These viruses constituted a group with no serological relationship to reovirus or orbiviruses but serologically related to one another (Welch and Twiehaus, 1973; Kapikian et al., 1976).

The International Committee on Taxonomy of Viruses (ICTV) classified the group as members of the reovirus family containing the reovirus, rotavirus and orbivirus genera (Fenner, 1976). The generic names 'rotavirus' and 'duovirus' have been proposed. 'Rotavirus', being the most commonly used, seems likely to be adopted by the ICTV (Kurstak et al., 1981).

The rotaviruses are further divided into several separate serotypes by neutralization (Thouless et al., 1977) or into two subgroups by enzyme linked immunosorbent assay (ELISA) (Zissis et al., 1978) and at least two serologically unrelated groups (Bridger et al., 1982). This classification is expanded in Section 1.2.6, which deals with rotavirus serology. The rotaviruses of each animal species are a separate serotype and human rotaviruses are subdivided into three or four serotypes (Thouless et al., 1977; Beards et al., 1980).

1.2.3 Clinical and pathogenic features of rotavirus infection

All rotaviruses cause gastroenteritis in their animal hosts. The severity of disease is very variable, ranging from sub-clinical to fatal in both children and young animals; and all infections are seasonal, occurring mainly in the winter months (Greenberg et al., 1981). Infections of adults have also been reported (Zissis et al., 1976; Bolivar et al., 1978; von Bonsdorff et al., 1978) but these are usually mild or symptomless. Infections in children under 6 months of age were rare (Flewett and Woode, 1978; Soenarto et al., 1981).

In humans, clinical symptoms of rotavirus gastroenteritis appear 48-72 hours after infection, sometimes preceded by vomiting. Diarrhoea, often with accompanying fever, is the main symptom (Shepherd et al., 1975; Tallett et al., 1977). This leads to dehydration and, in severe cases, high temperatures up to 40°C and possible hypernatremia. (Parrott, 1976). Fatalities appear to result from 2-4 days of vomiting and diarrhoea with subsequent dehydration and hypernatremia (Middleton et al., 1976). Rehydration with glucose electrolyte solutions is the most effective treatment and the course of diarrhoea then usually lasts 5-8 days before symptoms disappear. Other viruses or bacteria are often associated with fatal cases of gastroenteritis, but it is not clear whether they contribute to the cause of

1.2.3 (Cont'd).

death. Subclinical infections with rotavirus are also common as denoted by an antibody response but no symptoms.

Work with gnotobiotic calves (Mebus et al., 1969; 1971; Woode and Bridger, 1975) revealed that diarrhoea developed in 18-24 hours after infection and most calves became depressed and lost weight. If diarrhoea is prolonged, dehydration can cause death after 4-7 days and pneumonia has been found to be a common contributory factor to deaths which occurred 2-3 weeks after infection (Woode and Crouch, 1978). Recovery of severely ill calves is possible when fed with a glucose and saline mixture instead of milk (Woode and Crouch, 1978).

Infected piglets react similarly to calves, showing depression, anorexia, reluctance to move, vomiting after 18-24 hours and diarrhoea a few hours later (Woode et al., 1976b). Clinical symptoms regressed after 4-6 days although diarrhoea often lasted for 7-14 days. Recovery was aided when milk was replaced by water (Woode et al., 1976b). Lambs also suffered anorexia and diarrhoea at 11-18 hours after infection (Snodgrass et al., 1976). Foals and turkeys have shown clinical similarities to calves, pigs and lambs (Eugster and Whitford, 1978; McNulty et al., 1979).

1.2.3 (Cont'd).

Rotavirus infections have been reduced in severity to subclinical infections when the calf, pig or lamb is fed with colostrum (which contains maternal antibody) prior to infection (Woode et al., 1975; Snodgrass et al., 1977), and also when gnotobiotic piglets have been inoculated with rotavirus from children, foals or lambs (Bridger et al., 1975; Woode et al., 1976b. This latter observation is important as it demonstrates at least some species specificity of rotavirus infection.

Most of the pathogenic effect of rotavirus arises from replication in the small intestine, although rotaviruses have been found in the lungs and mesenteric lymph nodes of an infected calf (Mebus et al., 1971b) and rotavirus antigen has been detected in the colon and caecum of infected lambs (Snodgrass et al., 1977).

Rotavirus infects the epithelial cells of the absorptive portion of the villi, but not the crypt cells. The villi become markedly shortened or stunted and the columnar brush bordered epithelium is lost from their tips and replaced with cuboidal or squamous cells without a brush border, and an increased number of rounded cells are found in the lamina propria (Bishop et al., 1973; Barnes and Townsey, 1973). Thin section electron microscopy revealed that many of the cells were normal, but in discreet areas the enterocytes were vacuolated with damaged or necrotic

1.2.3 (Cont'd).

microvilli and contained rotavirus antigen (Davidson et al., 1975). The infected cells migrate to the tip of each villus, where they are shed, and are replaced with immature cuboidal cells from the crypt which apparently have no rotavirus receptors (Snodgrass et al., 1977) as reinfection of calves shortly after cessation of diarrhoea failed to produce clinical disease (Mebus et al., 1971b). Examination of late stages of infection show that the stunting of villi has resulted in fusion, leaving a flat avillous mucosa in some areas of the small intestine (Pearson and McNulty, 1977; Lecce and King, 1978). Despite this complete destruction of villi, regeneration in surviving animals was rapid and the intestine was usually normal within 10 days.

The intestinal lumen in normal conditions receives fluid from the diet and also from gastrointestinal secretions, and the intestine generally reabsorbs about 95% of the fluid in the intestinal lumen. Both increased secretion of fluid into the lumen, and decreased reabsorption can result in diarrhoea and dehydration (Desjeux et al., 1979). In rotavirus-induced gastroenteritis, both of these occur (Flewett and Woode, 1978). During the diarrhoeic phase, the villous epithelium resembles crypt epithelium and the villous tip cells contain less disaccharide and have a decreased ability to synthesise disaccharides, absorb glucose and galactose and to utilise lactose. Lactose and other

1.2.3 (Cont'd).

disaccharides remain in the lumen of the bowel, creating a high osmotic pressure which attracts body fluid into the lumen and prevents absorption of water from faeces (Flewett, 1977). The lactose therefore exerts a positive dehydrating effect which can be counteracted by replacing milk and other disaccharide sources with dilute electrolyte solution (Flewett and Woode, 1978). These immature villous cells have an impaired glucose coupled sodium transport system and adenyl cyclase activity is not increased (Hamilton et al., 1975). Furthermore, the inflammatory response associated with the infection causes increased peristaltic activity. This results in faster passage of food allowing insufficient time for digestion and the absorption of water by the villous cells (McNulty, 1978).

1.2.4 Immunity

The presence of rotavirus antibody in farm animals is extremely common. In a study of Scottish sheep, 38% of adult sheep and 56% of neonates were found to contain serum antibody to rotavirus (Snodgrass et al., 1977). In Canada, 79% of cattle studied were found to contain rotavirus antibody and a survey in Britain of 59 herds with a history of diarrhoea showed that all of them were serologically positive. Of those cattle studied in detail, more than 90% had neutralizing antibody titres of greater than 40 and 58%

1.2.4 (Cont'd).

had titres of 320 or more (Flewett and Woode, 1978). The antibody levels in many cattle appear to be too low to protect them from infection or to provide sufficient antibody in their colostrum and milk to protect the young calves (Kurstak et al., 1981). Colostral antibody appeared to be important in protection of young animals against disease (Bridger and Woode, 1975; McNulty et al., 1976; Lecce et al., 1976; Snodgrass and Wells, 1976, 1978), but antibody against rotavirus appeared only to be secreted in large quantities in first day colostrum and to decrease thereafter leaving most animals susceptible to infection by 3 days after parturition (Woode et al., 1975). Rotavirus induced gastroenteritis in calves most often occurs within the first seven days of life (Flewett and Woode, 1978). Animals are found to be protected by feeding with colostrum, especially continuous feeding of relatively small quantities (McNulty et al., 1976); Snodgrass and Wells, 1978). The protection is not entirely type specific-piglets can be protected from infection with porcine rotavirus by feeding with bovine colostrum (Bridger and Brown, 1981), and these protected piglets which suffered a sub-clinical infection were then resistant to further infection by porcine rotavirus. Passive immunisation by feeding animals with purified immunoglobulin with anti-rotavirus activity has also been shown to protect against disease (Lecce et al., 1976; Snodgrass et al., 1977). Lambs can be protected by human IgG,

1.2.4 (Cont'd.)

so cross-species protection also appears to be effective (Snodgrass et al., 1977).

The most important factor in natural protection does not appear to be serum antibody, but rather local immunity in the gut produced mainly by secretory IgA antibodies (Mebus et al., 1973).

Immunity in humans has been studied in some detail. Second attacks of rotavirus infection have been shown to be very rare (Middleton, 1977), suggesting that immunity prevails until adulthood. The presence of serum antibody varies with age. At birth, 73-80% of babies possess serum antibody, but this level of immunity declines becoming almost undetectable at 3 months and antibody levels begin to rise again at about 6 months. 50-90% of children aged 18 months possess antibody and 99.9% of adults in a small sample were serologically positive for rotavirus (Kapikian et al., 1975; 1976b). Immunity is highest at 6 years of age and slowly drops, becoming undetectable in people older than 70 years (Elias, 1977). However, serum antibody does not necessarily prevent infection, and breast fed babies do not get full protection from maternally derived colostrum antibody. For instance, in one survey of a ward, children with rotavirus infection consisted of 11 breast fed and 18 bottle fed babies (Chrystie et al., 1975). Human colostrum has been shown

1.2.4 (Cont'd).

to contain antibody, which is likely to be IgA (Thouless et al., 1977b; Kurstak et al., 1981).

During rotavirus infection, antibody is detectable within 2 days by IF (Davidson et al., 1975) and 2-4 days by IEM (Flewett et al., 1974). IgM is found first and then replaced by IgG (Konno et al., 1975). Complement fixing antibodies diminish fast after infection while neutralizing antibodies persist (Elias, 1977). IgA can be detected in faeces, colostrum and sera (Watanabe et al., 1978).

1.2.5 Diagnosis

Rotavirus infections were initially diagnosed by electron microscopy (EM). Bishop et al. (1973) used EM of thin sections of duodenal biopsies, and Flewett et al. (1973, 1974b) first used negative contrast to show the presence of rotaviruses in concentrated stool samples. Crude, unpurified stool suspensions have usually been found to be adequate for the latter method, which is now the most commonly used diagnostic procedure. Immune electron microscopy (IEM), which involves mixing virus and antibody and detecting the aggregates or using antibody coated EM grids to capture virus, is also used and can be more sensitive than direct negative staining which only easily detects virus at concentrations greater than 10^8 /ml (Flewett, 1978). However, the characteristic rotavirus morphology is less easily recognised

1.2.5 (Cont'd.)

in IEM as images of the particles are somewhat obscured by antibody.

Middleton et al. (1974) and Davidson et al. (1975) detected rotavirus antigen in biopsies and duodenal cells by indirect immunofluorescence, which was also used to determine the presence of antibody in serum after infection. Immunofluorescence has been widely used for research and diagnostic purposes (Bridger and Woode, 1975; Banatvala et al., 1975; Bryden et al., 1977; Thouless et al., 1977). It is reproducible and sensitive but is not easy when large numbers of samples are involved (Kurstak et al., 1981). A similar, recently developed technique for detection of rotavirus antigens and antibodies is immunoperoxidase staining. The marker antibody is coupled to horseradish peroxidase and immune complexes are stained with diaminobenzidine and visualised under a light microscope (Sternberger, 1979). Virus detection at the ultrastructural level is easier with immunoperoxidase than IF, as the staining is very discreet (Kurstak et al., 1981).

Other methods have also been developed for rotavirus diagnosis with emphasis on low cost and simplicity. Kapikian et al. (1974) detected rotavirus antigen in stool suspensions by a complement fixation (CF) test, but this technique is seldom used as frequent anticomplementary

1.2.5 (Cont'd.)

effects of faecal samples reduce the sensitivity of the test (Kurstak et al., 1981).

Counter immunoelectrophoresis (CIEP) allows rapid examination of large numbers of specimens, but is less sensitive than EM (Middleton et al., 1976; Spence et al., 1975).

Solid phase radioimmunoassay (RIA) is a more recently developed technique which is more sensitive than EM (Middleton et al., 1976; Kalica et al., 1977). It has now been superseded by an enzyme linked immunosorbent assay (ELISA), which is inexpensive and reproducible. Both techniques use polystyrene wells coated with antibody to which antigen is added. Immune complexes are detected by ¹²⁵I-labelled antibody in RIA and enzyme-labelled antibody in ELISA. The enzyme is most usually alkaline phosphatase, which forms a yellow colour on reaction with p-nitrophenol. Enzyme-linked antibody is more stable, more active and safer than ¹²⁵I-labelled antibody. ELISA has been used for detection of rotaviruses (Volken et al., 1977) and for the study of rotavirus epidemiology and differentiation of subtypes (Volken et al., 1978; 1978b). It is rapid, reproducible, quantitative and up to 100 times more sensitive than EM, IF, CF or CIEP (Ellens et al., 1978) and is now probably the principal diagnostic method.

1.2.5 (Cont'd).

A number of techniques for detecting rotavirus haemagglutinin have also been developed. Haemagglutination was achieved with calf and simian rotavirus complete particles, but not with single shelled particles (Fauvel et al., 1978; Kalica et al., 1978) and was inhibited by type-specific rotavirus antiserum. A high concentration of rotavirus particles (probably greater than 10^{12} /ml) seems to be necessary for successful use of this technique. A modification termed immune adherence haemagglutination assay (IAHA) has been developed and appears to be comparable to ELISA (Kapikian et al., 1978; 1981). IAHA involves coating of polystyrene wells with antibody (or antigen) and adding antigen (or antibody) to this. Immune complexes are recognised by addition of fresh complement followed by human erythrocytes and haemagglutination is scored 1-3 h later. Both IAHA and ELISA appear to have subgroup and not serotype specificity, while HI was thought to be serotype specific (Fauvel et al., 1978). IAHA therefore does not recognise the haemagglutinin, which is merely used as the indicator for binding erythrocytes, but recognises a different viral antigen involved in subgrouping. Genetic experiments have identified different antigens involved in determining serotype and subgroup (Section 1.3.9), and the use of monospecific and/or monoclonal antisera in the future will make identification of specific antigens possible, thereby diminishing the current confusion.

1.2.5 (Cont'd).

Plaque assay and plaque reduction (neutralization) with antisera are only applicable to tissue culture-adapted viruses but are the most accurate methods available as they give information on the titre of virus or antibody.

Techniques such as IEM, immunofluorescence, immunoperoxidase staining, complement fixation and RIA detect group-specific rotavirus antigens, i.e. one antiserum will react with all rotavirus serotypes and will usually not distinguish between them while ELISA, IAHA, HI and neutralization (plaque reduction) detect different subgroups or serotypes and can therefore distinguish between different viruses. ELISA and IAHA are subgroup-specific, while neutralization and HI are type-specific. These terms are explained in Section 1.2.6 on rotavirus serology. In IEM, double shelled particles are only agglutinated by type-specific antiserum, while single shelled particles will react with all antisera against rotavirus (Bridger, 1978).

1.2.6 Serology

Rotaviruses are probably antigenically distinct from other members of the reovirus family as there is no serological relationship between calf rotavirus and either reovirus or bluetongue virus by neutralization or immunofluorescence (Fernellius et al., 1972; Welch and Twiehaus, 1973). Later, Kapikian et al. (1974, 1975, 1976) used a complement fixation

1.2.6 (Cont'd)

test to show that several rotaviruses had no antigenic relationship with the three reovirus serotypes or with any of twenty orbiviruses. A separate genus for rotavirus has been proposed as a result of this combined evidence, and from other biochemical properties of the virus (Sections 1.3.1 and 1.3.3).

Rotaviruses from several animal species are serologically related to each other. Flewett et al. (1974) demonstrated a serological relationship between calf and human rotavirus by IEM, and Kapikian et al. (1975, 1976) confirmed the relationship for human, calf, simian (SA-11), murine (EDIM) rotaviruses and the 'O' agent using CF. Woode et al. (1976) also showed that these and pig, lamb, rabbit and foal rotaviruses cross-reacted by IEM, IF, CF and gel diffusion (GD) and suggested that these techniques all recognised rotavirus group antigens. By neutralization, Woode et al. (1976) and Thouless et al. (1977) detected quantitative serological differences between them, thereby demonstrating the presence of type-specific antigens in rotaviruses. The rotaviruses from each animal species studied constitute a separate serotype (Thouless et al., 1977), and at least three serotypes of human rotavirus have been identified by neutralization (Flewett et al., 1978; Thouless et al., 1978; Beards et al., 1980). Experiments to subdivide rotaviruses from other animal species into more than one serotype have not yet been carried out.

1.2.6 (Cont'd.)

Some cross-reactions were observed between some convalescent antisera from gastroenteritis and rotaviruses from other animals (i.e. different serotypes) in neutralization tests (Woode et al., 1976), which could reflect the fact that animals are sometimes cross infected with other rotavirus serotypes, as described in Section 1.2.3.

Two different types of human rotavirus were identified by ELISA and IAHA (Bishai et al., 1979; Zissis and Lambert, 1980; Zissis et al., 1981; Kapikian et al., 1981), and these were thought to correlate with the two major human rotavirus serotypes found by neutralization (Beards et al., 1980). However, ELISA and IAHA have recently been shown to divide viruses into subgroups rather than serotypes (Kapikian et al., 1981), and most animal rotaviruses fall into the same subgroup as human rotavirus type 1 (Kapikian et al., 1981). The ELISA assay recognises an antigen other than the neutralization antigen (Kalica et al., 1981), so the subgroup and the serotype are completely different properties of rotavirus.

Two porcine rotavirus isolates and an avian isolate have recently been found that are serologically unrelated to other rotaviruses at least by IF or IEM (Saif et al., 1980; McNulty et al., 1981; Bridger et al., 1982). Morphological studies showed that these isolates were identical to other rotaviruses and, like them, replicated in villous epithelial

1.2.6 (Cont'd).

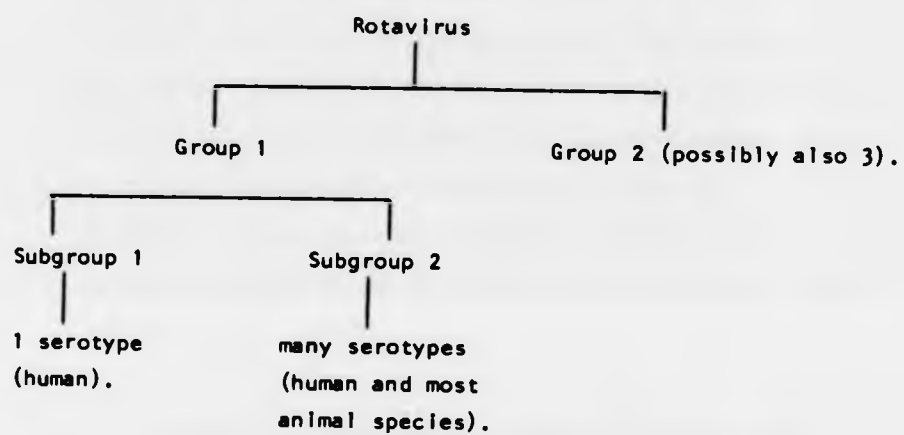
cells. The RNA pattern of the avian isolate and a porcine isolate was rotavirus-like but had a characteristic pattern different to the basic pattern common to other rotaviruses (McNulty et al., 1981; Bridger et al., 1982). Preliminary tests by McNulty have found no serological relationship between the avian and porcine isolates, so there may be at least two new serological groups (Bridger et al., 1982). Other reovirus genera are also subdivided into serologically distinct groups (Section 1.1).

The classification of rotaviruses is still incomplete, but they will probably be classified into at least two serologically unrelated groups lacking a common group antigen, with most viruses falling into a single group which can be subdivided into two subgroups by ELISA and several serotypes by neutralization (fig. 2).

Virus preparations also contain rotavirus antigenic subunits and tubular structures. Both of these appear to be aggregations of inner capsid polypeptides, represent the viral group antigen(s) and indicate over-production of certain viral polypeptides (Mathan et al., 1977; Kimura, 1981).

Fig.2b

Classification of the rotavirus genus



1.2.7 Morphology

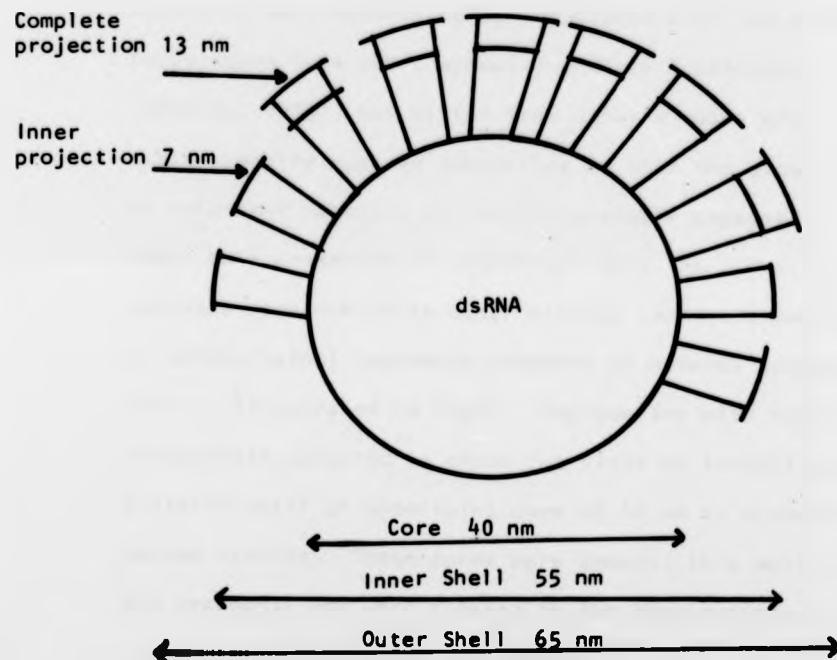
The early observations of rotavirus (Bishop et al., 1973) suggested that they resembled the orbiviruses.

Flewett et al., (1973) described the virus particles as 75-84 nm in diameter and similar to reoviruses with a double layered capsid. Bishop et al. (1974) also saw single shelled particles 60 nm in diameter and 'empty' capsids as well as complete particles. Middleton et al. (1974) suggested that some single shelled particles seen in cells were surrounded by a "pseudoenvelope". From later morphogenetic observations, these are probably immature particles (McNulty, 1979). Tubular structures of 54-100 nm in diameter have also been found (Holmes et al., 1975).

Structural studies were extended by Flewett et al. (1974b) who described a central core of 36-38 nm from which radiated cylindrical capsomers with an outer layer of shorter capsomers in complete particles (Fig.3). Complete particles had a diameter of 63 nm and were smooth in appearance, whereas the single shelled particles had scalloped edges and were only 55 nm in diameter. All rotaviruses studied are morphologically identical (Woode et al., 1976; Kapikian et al., 1976).

Detailed ultrastructural studies by analysis of chemical degradation of the viruses and electron

Fig.3 Diagram of rotavirus structure
(from Almeida, 1979)

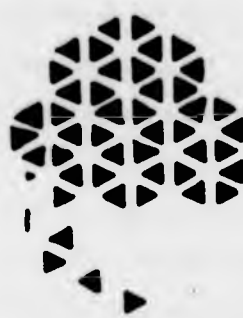


1.2.7 (Cont'd)

microscopy have been undertaken by many groups (Almeida, 1979; Martin et al., 1975); Palmer et al., 1977; Stannard and Schoub, 1977; Esparza and Gil, 1978). Viruses held for 2 days at 37°C in the presence of trypsin disintegrated and what remained were arrays of a single type of subunit in a lattice consisting of five or six subunits, each subunit being associated with two others. Rotaviruses have cubic symmetry and are icosahedral (Almeida, 1979), but differ from other viruses with cubic symmetry such as adenovirus in that the groupings of rotavirus subunits did not form stable capsomers. Thus, on degradation of rotavirus, only the structural subunits remained while other viruses tend to break down to morphological capsomers composed of several subunits. This is illustrated in Fig.4. Degradation with sodium thiocyanate appeared to erode the virus by solubilizing proteins until an underlying core of 40 nm in diameter became visible. These cores were smooth, thin walled and hexagonal and were similar to the reovirus cores recognised by Luftig et al. (1972).

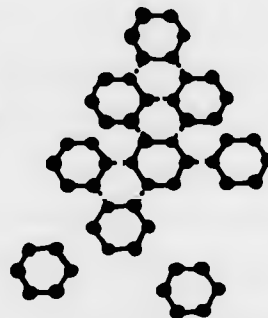
Electron microscopic analyses to determine the number and arrangement of structural subunits have been undertaken by several groups. Martin et al. (1975) saw only single shelled particles and suggested that they were composed of 540 subunits arranged into trimers

Fig.4 Capsid structure of degraded viruses
(from Almeida, 1979)



a)

Capsid structure of
rotavirus capsid
(single capsids)



b)

Capsid structure of
herpes virus
(hexamers)

1.2.7 (Cont'd.)

giving 180 structural subunits. These were arranged into 20 groups of 6, and 12 groups of 5 forming an icosahedron with the pentamers at the apices. Rotavirus thus followed the principles outlined by Caspar and Klug (1962) for icosahedral viruses. With a triangulation number $T = 3$, the virus had $60T$ (180) subunits and $10T + 2$ (32) vertices. The virus was also described as having a secondary T of 9 (making 540 subunits). Palmer et al. (1977) described the arrangement of the central core, which also has characteristics of an icosahedron. The inner capsid layer was described as similar to that shown by Martin et al. (1975) and consistent with the arrangement of structural subunits suggested for bluetongue and reovirus (Palmer et al., 1977; Palmer and Martin, 1977). Palmer et al. described the rotavirus outer layer as a continuous covering with a clearly defined margin. Stannard and Schoub (1977) postulated that the inner capsid had icosahedral symmetry and consisted of 180 structural subunits arranged in open lattice formation around 92 holes, i.e. $T = 9$. The 12 holes at the apices were surrounded by 5 capsomers and the other 80 spaces in the lattice were surrounded by 6 capsomers. The outer layer was also shown to have a honeycomb-like lattice and attached to the projections of the inner layer by short radial septa. Esparza and Gil (1978) agreed with Stannard and Schoub on the arrangement of morphological subunits

1.2.7 (Cont'd).

but suggested 162 rather than 92 holes and therefore involving 320 trimeric subunits and a triangulation number of $T = 16$. Roseto et al. (1979) examined freeze dried rotavirus particles and counted 132 capsomers arranged with $T = 13$ symmetry, which gave the virus a skewed icosahedral symmetry. The outer capsid appeared as a smooth covering with small holes, corresponding to those in the inner capsid. Table 2 outlines the various theories of rotavirus structure as well as proposals of reovirus structure for comparison.

TABLE 2

CAPSID STRUCTURE OF REOVIRUSES

(from McNulty, 1979)

GENUS	PROPOSED STRUCTURE	REFERENCE
ROTAVIRUS	a) 32 capsomers arranged with T=3 primary symmetry and T=9 secondary symmetry	Palmer <u>et al.</u> (1977) Martin <u>et al.</u> (1975)
	b) 180 capsomers arranged as an open mesh around 92 holes (80 surrounded by 6 capsomers, +12 surrounded by 5). T=9 primary symmetry.	Stannard and Schoub (1977).
	c) 320 capsomers arranged as open mesh round 162 holes. T = 16.	Esparza and Gil (1978).
	d) 132 capsomers, T = 13.	Roseto <u>et al.</u> (1979)
REOVIRUS	a) 92 hollow or columnar capsomers.	Jordan & Mayor (1962) Vasquez & Tournier (1962).
	b) 180 solid capsomers arranged around 92 holes.	Vasquez & Tournier (1964)
	c) 127 capsomers which are truncated pyramids.	Amando <u>et al.</u> (1971) Luftig <u>et al.</u> (1972)
	d) Probably 32 large capsomers arranged with T = 3 primary symmetry.	Palmer & Martin (1975)
ORBIVIRUS	a) 32 capsomers arranged with T = 3 primary symmetry	Els & Vorwoerd (1969) Murphy <u>et al.</u> (1978).
	b) 92 hollow tubular capsomers icosahedral capsid.	Owen & Munz (1966) Studdert <u>et al.</u> (1966)

1.3 Molecular Biology of Rotavirus and Reovirus

The molecular biology of reovirus has been studied in great detail, both as the prototype of the reovirus family and as a good model system for eucaryotic genomes. Knowledge of rotavirus biochemistry is much less advanced, mainly because intense research into this virus group only began in the early 1970's when their medical implications first came to light. As rotaviruses have shown great similarity to reoviruses in terms of molecular biology, reovirus has been used as a comparative model system for rotavirus research and similarly many methods adapted for the reovirus system have also been found to be useful for rotaviruses. It is therefore appropriate to explain in the next section the molecular biology of reoviruses and then to compare the rotavirus system to this. The reovirus chosen as a model is mammalian reovirus serotype 3.

1.3.1 RNA and variation among RNA's

1.3.1.1 Reovirus Double Stranded RNA

Approximately 15% of the reovirus particle mass is RNA, and 75% of this is double stranded (Silverstein, 1976). The double stranded RNA genome is the distinguishing characteristic of the reovirus family and it represented the first demonstration of the occurrence of dsRNA in nature (Gomatos et al., 1962). The fact that reovirus contained dsRNA was demonstrated by its resistance to

1.3.1.1 (Cont'd).

DNAase but not RNAase under low salt conditions and by its orthochromatic staining with acridine orange which resulted in pale green fluorescence and not the metachromatic bright red fluorescence indicative of single stranded nucleic acids (Gomatos et al., 1962). Further evidence of the RNA's double stranded character includes its sharp melting profile, its sensitivity to RNAase III but partial resistance to other RNAases (Bellamy et al., 1967), the absence of a hyperchromic effect on reaction with formaldehyde, equal ratios of adenine :uridine and guanine:cytosine (Gomatos and Tamm, 1963) and its density of 1.61 g/ml in CsSO₄ (Shatkin, 1965). X-ray diffraction data has also indicated that the reovirus genome is double stranded RNA.

The reovirus genome was found to be segmented after many attempts to extract the RNA in a single molecule resulted in several small pieces. Subsequently, Bellamy et al. (1967) demonstrated that these segments always fell into three discreet size classes. These three size classes were heterogeneous, and further analysis showed that there were ten molecular species in equimolar proportions with a combined molecular weight of about 15×10^6 daltons, which was the expected size of the genome. (Shatkin et al., 1968). The possibility that these segments arose as a result of breakage of 'weak

1.3.1.1 (Cont'd).

spots" along the genome was eliminated because each segment is fully base paired at each end (Muthukrishnan & Shatkin et al. 1974), each 5' end is capped (Furuchi et al., 1975) and 3' end labelling with K10₄ and ³H-NaBH₄ produces ten segments all labelled at the 3' terminus (Millward and Graham, 1970). Three factor crosses also show random segregation of RNA species indicating that they are not linked (Cross and Fields, 1976). As the transcriptase is part of the virion structure (section 1.3.3.2) and as the genes are transcribed simultaneously and at independent rates, the RNA segments are also unlikely to be covalently linked in virus particles. However, each virus particle contains 1 copy of each of 10 RNA species, so the RNA's must be assembled into virus particles by some highly specific mechanism, which may involve non-covalent linkage of genes by protein molecules. Kavenoff et al. (1975) have released reovirus RNA in a spider-like arrangement with all the RNA segments connected at a common focus, which may mean that they are sometimes linked in virus particles.

The RNA genome of all members of the reovirus family is segmented and in reovirus and rotavirus each specific segment has been shown (or calculated by the coding potential and gene coding assignments) to be monocistronic (Bishop, 1977; McCrae and Joklik, 1978; Smith et al., 1980; Dyal-Smith and Holmes, 1981).

1.3.1.1 (Cont'd.)

Usually the entire length of each segment is transcribed and translated (Bishop, 1977). Each of the serologically unrelated groups of the reovirus family (fig.2) has a distinctive RNA pattern on polyacrylamide gel electrophoresis (PAGE) (Stanley, 1981) and most of these groups are divided into serotypes with the same basic RNA pattern but showing substantial heterogeneity in the migration of individual segments (Ramig et al., 1977; Hrady et al., 1979; Rodger et al., 1975; Kalica et al., 1978; Rodger et al., 1981). This heterogeneity has been useful in determining reassortment of the RNA's between serotypes of reoviruses and rotaviruses (Cross and Fields, 1976; Greenberg et al., 1981b; Clarke and McCrae, 1982). Other members of the family also undergo genome reassortment (Stanley, 1981). This property has been exploited in genetic experiments to determine the gene functions of different segments, as described in section 1.3.5.

1.3.1.2 Reovirus Oligonucleotides

About 25% of the total RNA in reovirus particles is single stranded RNA (Shatkin and Sipe, 1968; Bellamy et al., 1972). This single stranded RNA is in the form of short molecules with ppp at their 5' ends - one third of these are oligoadenylates with only adenosine, and the rest are GC_{OH}, GCU_{OH}, GCUA_{OH}.

1.3.1.2 (Cont'd).

GCUA(U)₁₋₄U_{OH} or GCUA(A)₁₋₃A_{OH} or the 5' G-terminated oligonucleotides (Nichols et al., 1972; Bellamy et al., 1972). The sequence of these is comparable to the 5' terminus of reovirus plus strands, all of which have the sequence GCUA at their 5' end (Li et al., 1980) and these oligonucleotides are therefore clearly products of abortive transcription. Possible mechanisms for the production of the oligoadenylates and the 5' G-terminated oligonucleotides are outlined in section 1.3.3.1.2.

1.3.1.3 Sequences of Reovirus RNA

There have been several recent reports on the sequences of reovirus genes which are of interest in investigating the controls that regulate translation. Each reovirus mRNA is translated at a different frequency, and this appears to be controlled by the sequences of the genes themselves (Joklik, 1981). The possibility that the ten reovirus RNA segments had complementary ends which allowed all of them to associate together and enabled the virus to incorporate the appropriate RNA's during morphogenesis was quickly ruled out by showing that the terminal dinucleotide sequences of all the reovirus genes were identical (Bannerjee and Shatkin, 1971; Miura et al., 1974). More comprehensive sequence analysis of the 5' terminal sequences of six reovirus mRNA's was

1.3.1.3 (Cont'd).

undertaken by Kozak and Shatkin (Kozak and Shatkin, 1977, 1977b; Kozak, 1977), and they noted little similarity between them, except that they all had a 5' terminal sequence of GCUA. The 5' ends of these genes had been isolated by RNAase treatment of mRNA's bound to either 40S ribosome subunits so that only the terminal 50-60 residues were protected from digestion or to 80S ribosomes which protected about half of these residues, including the initiation codon AUG located 15-33 residues from the 5' end. Sequencing also showed that the mRNA's possess very little secondary structure - e.g. the S1 gene of reovirus serotype 2 has only one possible hairpin loop containing 3 termination codons (Kozak and Shatkin, 1977b; Li et al., 1980; Fig.5), while the S2 gene does not have any loop and other mRNA's may also lack it (Joklik, 1981). All mRNA's have a short 5' sequence complementary to the 3' end of 18S ribosomal RNA known as a Shine and Dalgarno sequence (Shine and Dalgarno, 1974; Kozak and Shatkin, 1978). Each mRNA has a different Shine and Dalgarno sequence. The mRNA's also have 6-10 residues of complementarity near the 5' and 3' ends which allow the two ends to associate (Li et al., 1980; Fig.5), so it appears that sequences upstream from the initiation codon can associate either with their own 3' terminus or with 3' sequences of 18S ribosomal RNA.

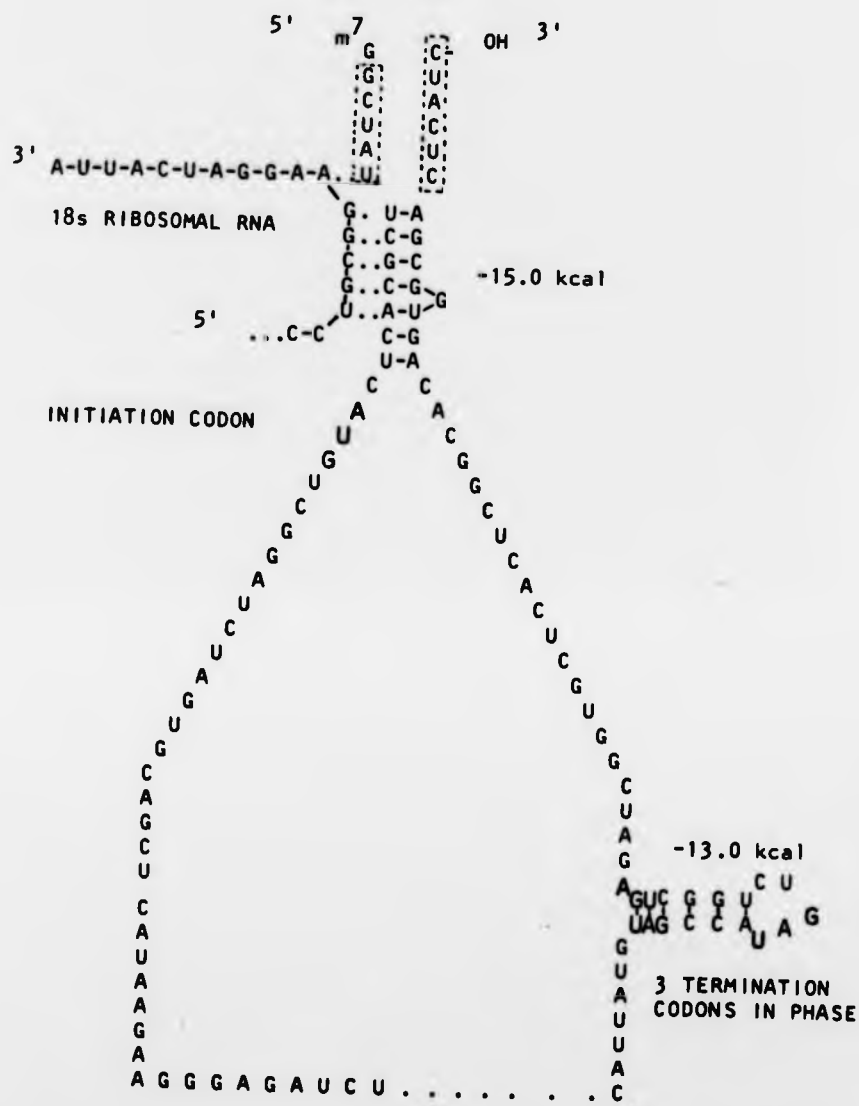


FIG.5. Possible partial secondary structure of the s1 mRNA of reovirus serotype 2. (Li *et al.*, 1980).

1.3.1.3 (Cont'd).

This may be part of a control mechanism to regulate the frequency of translation of each gene. McCrae (1981) and Li et al. (1980b) showed that the terminal sequences of the reovirus mRNA segment S2 are identical to the plus strand of dsRNA. The ends are not modified as in the case of Influenza virus mRNA which is a prematurely terminated copy of the minus strand, while positive sense templates are full length copies (Hay et al. 1977). McCrae (1981) showed that the ends of all 10 mRNA's were identical to the plus strand of dsRNA.

The reovirus S1 gene codes for the reovirus type specific antigen $\sigma 1$, and so a comparative study of the 3' ends of both strands of the S1 genes of three reovirus serotypes was undertaken to determine the genetic basis of this type-specificity and to identify conserved and variable regions (Li et al. 1980). The 3' end of the minus strand provided the sequence for the 5' end of the plus strand. About 80 bases of each end were sequenced, so the analysis included a substantial part of the coding region, which began about 15 bases from the 5' end and terminated about 40 bases from the 3' end (Fig.6). Comparison of the coding regions from the three serotypes revealed much dissimilarity, but the final ratio of neutral, charged and hydrophobic amino acids were similar in each case. As expected, the ends of the genes were

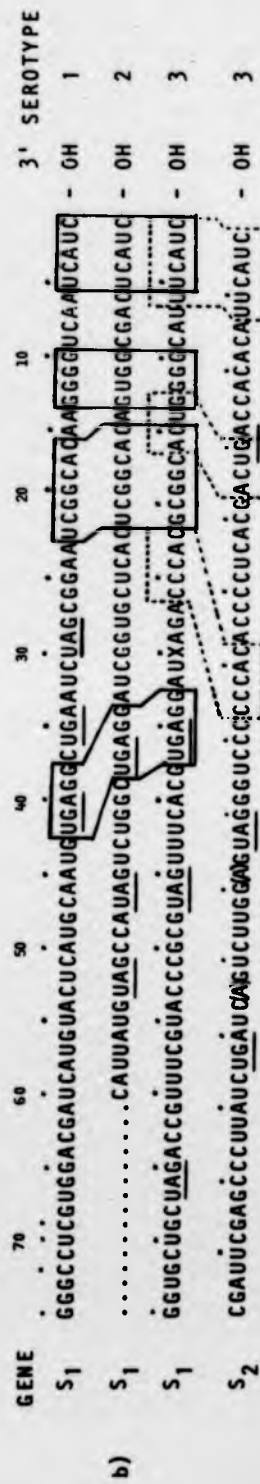
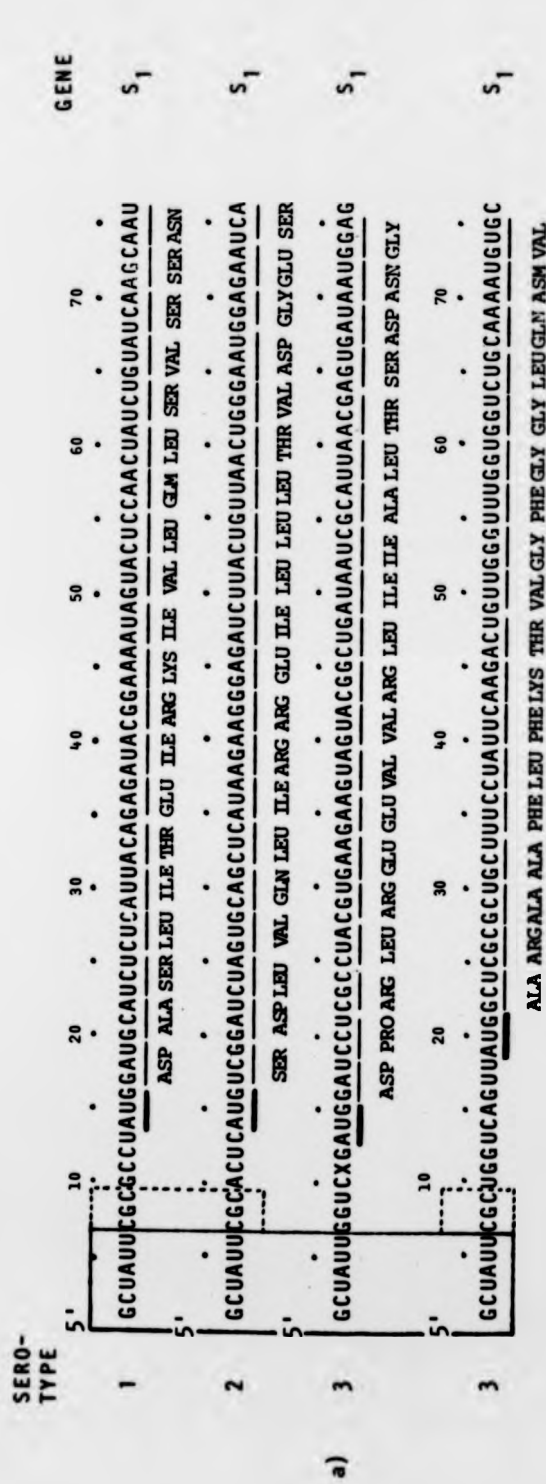


Fig. 6 Sequences of the 5' termini (A) and 3' termini (B) of the plus strands, that is, the mRNA's of the S1 genes of reovirus serotypes 1, 2 and 3 and of the S2 gene of reovirus serotype 3 (54).

(from Joklik, 1981).

1.3.1.3 (Cont'd).

more homologous - about 20 bases at the 3' ends showed substantial homology, while the 5' ends had 6-9 identical bases followed by 6-9 without homology before the initiation codon. The sequences of each of the L3, M3 and S2 genes from the three serotypes showed that these all had more serotype homology than S1 (Joklik, 1981). Each had identical sequences between the 5' ends and the initiation codons, identical coding regions for the first 10-20 codons and strong similarities at the 3' ends. Variation in the sequences of these genes thus reflected the variation in the proteins, although the proteins may also differ by post-translational modifications and secondary structure.

1.3.1.4 Rotavirus double stranded RNA

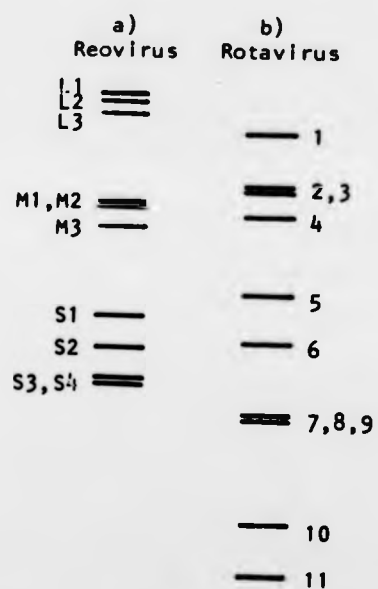
The characterisation of the rotavirus genome as double stranded RNA by Newman et al. (1975), Roger et al. (1975) and Schnagl and Holmes (1976) provided further evidence for classifying these viruses as part of the reovirus family. Rotavirus RNA was shown to be double stranded by its RNAase resistance, orcinol and diphenylamine reactions and thermal denaturation profile. PAGE analysis showed that the RNA was segmented and the segments were generally smaller in size than reovirus segments and did not fall into discreet size classes (Rodger et al., 1975; Newman et al., 1975). However, the eleven RNA segments formed an electrophoretic pattern which was characteristic of rotaviruses (McNulty, 1979).

1.3.1.4 (Cont'd).

The RNA from the following rotaviruses has the same basic electrophoretic pattern: calf (Newman et al., 1975; Rodger et al., 1975; Verly and Cohen, 1977; Kalica et al., 1978), human (Kalica et al., 1976; 1978; Espeijo et al., 1977; Schnagl and Holmes, 1976), pig (Todd and McNulty, 1976), lamb (Todd and McNulty, 1977), simian [SA-11] (Kalica et al., 1978), turkey and chicken (Todd et al., 1980). The molecular weight of the RNA segments ranges from about 2.2×10^6 daltons to 0.2×10^6 daltons giving a combined molecular weight of about 11×10^6 daltons, while 10 segments of reovirus RNA have a molecular weight of approximately 15×10^6 daltons and the three largest reovirus RNA segments are larger than the largest rotavirus RNA (see fig. 8). The antigenically distinct rotaviruses (see section 1.2.2) have eleven segments of rotavirus-like RNA, in the same size range as other rotaviruses but these form a distinct electrophoretic pattern to the rotaviruses described above (fig. 7, McNulty et al., 1981; Bridger et al., 1982).

The general RNA pattern on PAGE of the original rotavirus group is similar but, as in the case of reovirus, there is substantial heterogeneity in the migration of individual RNA segments, when RNA patterns from different rotavirus isolates are compared (Kalica et al., 1978; Rodger and Holmes, 1979; Todd et al., 1980). Rotaviruses

Fig.7 Schematic diagram comparing the relative sizes of reovirus and rotavirus genomic RNA (adapted from PAGE profiles shown in Todd *et al.* (1980)).



1.3.1.4 (Cont'd).

from different animal and avian species belong to different serotypes and because of the heterogeneity in the RNA patterns, it was thought that comparison of RNA profiles might serve as a useful alternative to serotyping. However, differences in RNA pattern do not appear to correlate to serotype so the significance of each different RNA pattern is not yet known. Matters are also complicated by diversity in the RNA pattern of isolates from the same animal species, including calves, humans, pigs and turkeys (Verly and Cohen, 1977; Kalica et al., 1978b; Todd et al., 1980; Espejo et al., 1980; Rodger et al., 1981). The epidemiological study of Rodger et al. (1981) identified 19 different electropherotypes of human rotaviruses and although different serotypes are known to exist within animal species, the number of human rotavirus serotypes identified is 3 or 4 rather than 19 (Flewett et al., 1978; Beards et al., 1980; Zissis et al., 1981). Therefore it seems that much, if not all, of the variation in migration of RNA is unrelated to serotype and cannot be used to classify strains. The best correlation found between RNA pattern and serological group is shown in the case of the two 'subgroups' identified in the ELISA assay (Zissis and Lambert, 1980, Kapikian et al., 1981). These correlate with the two main rotavirus electropherotypes which differ in the migration

1.3.1.4 (Cont'd).

of RNA segments 10 and 11 and are known as the 'short' and 'long' electropherotypes (Dyall-Smith and Holmes, 1981; Kalica et al., 1981b). However, one only finds these two basic variants and, while human rotaviruses are divided into two ELISA subgroups and the corresponding electropherotype, the rotaviruses from other animal species fit into one of these two groups (Kapiikian et al., 1981). Attempts to correlate neutralization-specific serotype with RNA migrational differences have been unsuccessful, possibly partly due to the fact that the gene(s) coding for type-specific neutralization (segment 5 or 9 described in section 1.3.4) migrate very closely with segment 7 and migrational differences in any one of these genes are difficult to detect. In any case, variation in RNA's from different serotypes may not be restricted to RNA's coding for the antigen in question. For example, although subgroup specificity is determined by gene segment 6 (Kalica et al., 1981), this segment does not show significant mobility variation while the two subgroups are characterised by the reversal in mobility of segments 10 and 11 described above.

Although routine analysis of serotypes cannot at present be achieved by analysis of RNA, the RNA profile of isolates may provide more specific information about

1.3.1.4 (Cont'd).

the origin and epidemiology of the virus as it can show persistence or variation of different isolates (Espejo et al., 1980; Rodger et al., 1981). For example, the epidemiological survey by Rodger et al., (1981) showed that the virus isolates tended to persist over a 12-24 month period before being succeeded by a new type, which in many cases was not a different serotype. Rodger et al. also showed a significant epidemiological difference between virus isolates recovered from neonates and those from young children, as isolates from neonates persisted in a hospital for several years while those from children changed frequently. This information would not have been available by serotyping as the new isolates were not usually different serotypes.

Study of variation in RNA's of different rotavirus isolates has been extended by Clarke and McCrae (1982), who analysed T₁ oligonucleotide patterns of each gene segment of several bovine rotavirus isolates. Their evidence suggested that the amount of variation in sequence was not correlated to changes in mobility, i.e. sequence changes at least as great as those found in segments showing variation in electrophoretic mobility were also detected in segments showing no such variation. Segments 4 and 11 (coding for VP4 and VP10) showed the greatest degree of sequence variation (even when there

1.3.1.4 (Cont'd.)

was no mobility change) while the other RNA segments, some of which show changes in mobility, were more highly conserved in sequence. Therefore, the electrophoretic mobility differences of RNA segments could be less significant than has been previously assumed. By analogy with influenza viruses (Palese and Schulman, 1976) these minor changes could be a result of antigenic 'drift', i.e. single mutations, while the major changes in sequence seen in gene segment 11 could be the result of antigenic 'shift', or gene reassortment between two viruses simultaneously infecting a single host. Clarke and McCrae (1982) have suggested gene reassortment as a method of producing the diversity seen in gene segment 11 because of the 5 isolates analysed, there are two distinct classes of segment 11 which are significantly different and evolution by antigenic drift in two separate viruses would be unlikely to produce similar major changes. Gene reassortment in rotaviruses has been shown to occur at high frequency in laboratory experiments (Matsuno *et al.*, 1980; Greenberg *et al.*, 1981b), and isolates from a single animal which contain two distinct viruses have also been found (Clarke and McCrae, 1982), so variation of rotaviruses in the wild as a result of gene reassortment is possible. Preliminary work on sequencing and cloning of the rotavirus genome was reported by McCrae and McCorquodale (1982b; 1983). The 3' terminal octanucleotides of the eleven plus strands and mRNA of calf and human rotavirus are identical, as are the 3' octanucleotides of the minus strands. Insertion of the genome segments into the plasmid PAT 153 and grown in E.coli has been successful but expression of these cloned sequences has yet to be determined.

1.3.2 Polypeptides, variation and antigens

1.3.2.1 Reovirus Polypeptides

Reovirus structural polypeptides were first studied by Loh and Shatkin (1968) and later by Smith et al. (1969), who devised the nomenclature. These authors identified 7 structural polypeptides, and this was later increased to nine by Both et al. (1975). The polypeptides are listed in Table 3. The polypeptide pattern is fairly simple, as will be discussed later, since most polypeptides are primary gene products and there are only two non-structural polypeptides. The structural proteins are associated with either the core or the outer shell, and the approximate number of molecules of each protein per virion has been estimated, as seen in table 3. The current status of each polypeptide is discussed below.

A. Core Proteins. The inner shell of reovirus is known as the core. This comprises about 30% of the protein mass, and contains major polypeptides λ_1 (105 molecules per virion), λ_2 (60 molecules per virion) and σ_2 (200 molecules per virion) as well as minor components λ_3 , μ_2 (of which there are less than 12 molecules per virion) and μ_1 (20 molecules per virion). The function and distribution of the three minor core proteins is unknown. None of the core proteins appear to be associated with dsRNA as empty particles (particles lacking dsRNA) have the same protein composition as

T A B L E 3 Reovirus Type 3 Polypeptides (From Joklik, 1981).

dsRNA (McCrae & Joklik 1978)	Protein Species (old nomenclature) (Zweerink et al., 1971)	Protein Species (new nomenclature) (McCrae & Joklik, 1978)	M.W.	% in virion	Approx. No. per Particle	Location
L3	λ_1	λ_1	155,000	15	105	Core
L2	λ_2	λ_2	150,000	11	90	Core
L1		λ_3	135,000	≤ 2	≤ 12	Core
M2	μ_1	μ_1	80,000	2	20	Core
M2	μ_2	μ_1c	72,000	35	550	Outer Shell
M1	μ_3	μ_2	70,000	≤ 2	≤ 12	Core
S1	σ_1	σ_1	42,000	1	24	Outer Shell
S2	σ_2	σ_2	38,000	7	200	Core
S4	σ_3	σ_3	34,000	28	900	Outer Shell
M3	σ_0 (μ_4)	μNS	75,000			
S3	μ_{2A}	σNS	36,000			

1.3.2.1 A. Core Proteins (Cont'd).

complete particles and, as the core cavity is only just large enough to accommodate the known mass of genomic dsRNA and the oligonucleotides always found in virions (section 1.3.1.2), only one of the minor polypeptides could fit into the space. No association of any of these minor proteins with dsRNA has been demonstrated.

Of the major polypeptides, $\lambda 2$ is the major component of the 12 projecting core spikes (White and Zweerink, 1976). Each spike has been shown by cross-linking to be a pentamer of $\lambda 2$ (Ralph et al., 1980). These spikes appear to project through the outer shell and to be exposed on the virion surface, as monoclonal antibody to $\lambda 2$ reacts strongly with complete virus particles (Hayes et al., 1981). The other two major core components ($\lambda 1$ and $\sigma 2$) probably form a network of capsomeres containing 1 molecule of $\lambda 1$ to 2 molecules of $\sigma 2$. $\lambda 1$ is more readily iodinated than $\sigma 2$ and may therefore be closer to the core surface (White and Zweerink, 1976). It has been suggested that all three of the major core polypeptides form part of the transcription enzyme complex (see section 1.3.3.1), but there is little evidence for this at present.

1.3.2.1 B. Outer Shell Proteins. The reovirus outer shell consists of three polypeptides - $\sigma 3$ (900 molecules per virion) $\mu 1c$ (550 molecules per virion) and $\sigma 1$ (24 molecules per virion). $\sigma 3$ and $\mu 1c$ between them comprise more than 60% of the total reovirus protein mass and provide the network of the outer shell, possibly in capsomeres of 1 molecule of $\mu 1c$ to 2 molecules of $\sigma 3$. These proteins have strong affinity for each other - more than 50% of each of these proteins in infected cells are always complexed together (Huismans and Joklik, 1976). Removal of $\sigma 3$ from the outer surface by chymotrypsin accentuates the appearance of the capsomer structure, and it has been suggested that $\sigma 3$ forms the outermost spikes and $\mu 1c$ the capsomers at the base of the spikes (Astell et al., 1972). The fact that subviral particles lack $\sigma 3$ but have a 12,000 dalton fragment of $\mu 1c$ and the fact that monoclonal antibodies to both $\sigma 3$ and $\sigma 1$ neutralize reovirus infectivity while those to $\mu 1c$ do not, suggest that $\sigma 3$ is the outermost polypeptide (Hayes et al., 1981). An unusual property of $\sigma 3$ is its strong affinity for dsRNA; the free form of $\sigma 3$ binds to poly I:poly C. The location of the minor polypeptide $\sigma 1$ is unclear, but it must be exposed as it is the most important polypeptide for infectivity and virulence, and is the neutralisation antigen and haemagglutinin. $\sigma 1$ may be close to (or on) the core spikes as antibody to $\lambda 2$, but not to

1.3.2.1 B. Outer Shell Proteins (Cont'd)

$\sigma 3$ or μlc , can prevent anti $(\alpha)\sigma 1$ binding to $\sigma 1$ (Lee et al., 1981b). The functions of $\sigma 1$ will be discussed in section 1.3.5.

Comparisons of reovirus polypeptides from the three serotypes revealed that $\sigma 1$ is the most type-specific by immune precipitation (Gaillard and Joklik, 1981), neutralisation (Hayes et al., 1981) and in its peptide map (Gentsch and Fields, 1981).

1.3.2.2 Comparisons of Reovirus polypeptides between the three serotypes. Ramig et al. (1977) compared polypeptides from each of the three serotypes by polyacrylamide gel analysis, and found substantial heterogeneity in migration of each of the polypeptides (Table 4). This was expected for outer shell polypeptides, and had previously been noted among bluetongue serotypes (De Villiers, 1974), but was somewhat surprising among core proteins which might be expected to be functionally conserved. This heterogeneity was analysed further by tryptic peptide mapping of outer capsid polypeptides (Gentsch and Fields, 1981). μlc displayed more conservation between the serotypes than expected (as this protein varies in its resistance to proteases (section 1.3.5), while $\sigma 3$ and $\sigma 1$ had both conserved and unique tryptic peptides. The degree of divergence

TABLE 4

Polypeptide heterogeneity of reovirus serotypes 1, 2 and 3: Summary of the distinguishable molecular weight differences. (from Ramig *et al.*, 1977).

Polypeptide	Comparison
λ_1	$1 \neq 2 = 3D$
λ_2	$1 \neq 2 = 3D$
λ_3	$1 \neq 2 = 3D$
$\mu 1$	$3D \neq 2 \neq 1 = 3A$
$\mu 1c$	$3D \neq 2 \neq 1 = 3A$
μNS	$1 \neq 2 = 3A = 3D$
$\sigma 1$	$2 = 3A \neq 1 \neq 3D$
$\sigma 2$	$1 = 2 \neq 3A = 3D$
σNS	$2 \neq 1 = 3A = 3D$
$\sigma 3$	$2 \neq 3A \neq 3D = 1$

1 = Serotype 1, 2 = Serotype 2, 3 = Serotype 3

D = Dearing Strain, A = Abney Strain.

\neq detectable difference in migration of polypeptides between serotypes by PAGE.

$=$ no detectable difference.

1.3.2.2 (Cont'd).

was slight enough to suggest a common ancestral source for the mammalian reoviruses with subsequent evolutionary divergence accounting for the changes.

1.3.2.3 Rotavirus Polypeptides

The number, location and origins of the structural polypeptides of rotavirus have been more difficult to elucidate than those of reovirus and there has been much controversy about the polypeptide pattern and nomenclature. As these viruses did not grow in tissue culture, earlier work identified structural polypeptides of naturally occurring viruses purified from faeces (Newman et al., 1975; Bridger and Woode, 1976; Rodger et al., 1975, 1977; Todd and McNulty, 1977; Kalica and Theodore, 1979). There were many differences in both the number and molecular weights of the polypeptides found in these studies, and the different nomenclature used by each group also caused considerable confusion. However, McNulty (1979) organised the polypeptides into a basic pattern. Table 5 shows the similarities and differences of the polypeptides found by the above groups, and is based on the profile and nomenclature of Rodger et al. (1977). Most of the differences are in the molecular weights of the polypeptides and in the number of minor polypeptides. There appeared to be general agreement that VP5, VP7, VP8 and possibly VP9, are outer shell polypeptides, while

TABLE 5

Rotavirus polypeptides identified by different groups (from McNulty, 1979)

ROTAVIRUS STRUCTURAL POLYPEPTIDES									
Viral Protein (VP)	CALF				LAMB	HUMAN		SA-11	
	Newman et al. (1975)	Bridger & Woode (1976)	Todd & McNulty (1977)	Rodger et al. (1975, 1977).		Todd & McNulty (1977)	Rodger et al. (1975, 1977)	Objeski et al. (1977)	Rodger et al. (1975, 1977).
VP1	135*	125	128	131	128	127	155	133	128
2	122	103	108	103	108	103	137	102	120
3	96	98	99	97	99	97	130	99	115
4			96	92	96	88	118	92	110
5		63		58	72	58		58	67
6	36	44	50 48	32	50 48	32	40	31	48
7				22	37	26	32	23	
8				16.5	30	21	24	18	
9				14.5				14	

Table 5 Cont'd. Overleaf.

TABLE 5 (Cont'd).

Viral Protein (VP)		ROTAVIRUS FROM INFECTED CELLS								
		CALF		Thouless (1979)						
		Matsuno & Mukoyama (1979)								
		1	0	CALF	PIG	LAMB	MOUSE	FOAL	RABBIT	HUMAN
VP1	130			135.3	130.6	136.5	133.4	135.5	135.5	130.0
2	115			95.1	99.3	95.5	94.4	94.4	96.6	96.6
3	97			85.9	91.2	83.6	83.8	83.8	85.1	89.1
4										
5	68			53.2	52.2	52.4	52.2	52.7	53.6	53.1
6	³⁴ 32.5			42.7	40.4	42.7	41.1	43.7	41.8	41.5
7	38			26.4	26.2	26.2	25.0	27.7	26.0	26.8
8	24.5			24.6	24.1	25.0	23.4	26.4	24.2	25.0

The nomenclature and profile are of Rodger et al., (1979).

1 = inner shell 0 = outer shell.

*The numbers refer to estimated molecular weights of the polypeptides.

1.3.2.3. (Cont'd).

the rest are found in single shelled particles. The failure of the early attempts (Newman et al., 1975; Bridger and Woode, 1976; Todd and McNulty, 1976) to detect many low molecular weight polypeptides in calf rotavirus, and of Kalica and Theodore (1979) to detect low molecular weight polypeptides in SA-11 (simian) rotavirus was probably a combination of poor resolution and low virus concentration. These viruses were mainly purified from faecal material, and the amount of protease present in the samples was not defined and, as rotavirus polypeptides are processed by proteases, this factor alone could have influenced the polypeptide pattern. Matsuno and Mukoyama (1979) and Thouless (1979) studied polypeptides of rotavirus grown in cells and labelled with ³⁵S-methionine. Thouless grew virus in the presence of 10 µg/ml trypsin and Matsuno and Mukoyama compared polypeptide patterns of rotaviruses grown in the absence of trypsin either untreated or treated with 10 µg/ml trypsin. These studies therefore characterised the products of protease-activated virus particles under known conditions and their results are included in Table 5. Several recent studies on rotavirus structural polypeptides (Clark et al., 1981; Estes et al., 1981; McCrae and Faulkner-Valle, 1981; Dyal-Smith and Holmes, 1981) have helped to provide a clearer picture of rotavirus structural proteins, which agrees with the pattern seen by

1.3.2.3 (Cont'd).

Thouless (1979). Their work will be discussed in detail in the next section and that on in vitro translation and processing of rotavirus polypeptides (1.3.4.2), but the current status of rotavirus polypeptides is summarised in Table 6.

1.3.2.4 Topography and function of rotavirus polypeptides

Rotavirus polypeptides can be separated into inner and outer protein shells (Table 6), as naturally occurring single shelled and double shelled particles are separable by CsCl density gradient centrifugation (Rodger et al., 1975) and treatment of virions with 1.5 mM EDTA specifically removes the viral outer shell proteins (Cohen, 1977). Current understanding of the topography and function of each of the proteins is limited. The information is outlined below.

Inner shell polypeptides. The inner shell comprises approximately 60-70% of the total protein mass (Novo and Esparza, 1981) and was made up of polypeptides VP1, VP2 and VP6. VP6 comprised approximately 80% of the protein mass of the inner shell, VP2 20% and VP1 1%. Novo and Esparza (1981) used ¹²⁵I labelling to show that VP6 was less readily iodinated than VP1 or VP2, and concluded that VP6 was partly obscured in virus particles by VP1 and VP2. They also estimated that there were 6 molecules of VP1 per virus particle, 140 of VP2 and 989 of VP6, and proposed a

Table 6Rotavirus proteins

<u>Protein species VP</u>	<u>Approximate molecular weight (see Chapter 4).</u>	<u>Location</u>
1	125,000	inner shell
2	98,000	inner shell
3	82,000	outer shell
4	86,000	non-structural
3*	78,000	inner shell
4*	76,000	inner shell
4.2	62,000	outer shell
5*	51,000	inner shell
5	50,000	non-structural
6	42,000	inner shell
7	38,000	non-structural
7.1	36,000	outer shell
7.2	33,000	outer shell
8	32,500	non-structural
9	32,000	non-structural
vpr7	31,000	non-structural
10	27,000	non-structural
10c	26,500	outer shell
11	24,500	non-structural
11c	23,000	?
12	20,000	non-structural

1.3.2.4 (Cont'd.)

model for the arrangement of inner shell polypeptides. In trypsin-treated virus some cleavage products of VP2, i.e. VP3*, VP4* and VP5* are also seen in small quantities (see Section 1.3.3.4.2). As is the case with reovirus, the virion polymerase and the methylating and capping enzymes are associated with one or all of the inner shell polypeptides as these functions are carried out by the single-shelled particles (Cohen, 1977; Section 3).

Outer shell polypeptides. The rotavirus outer shell comprises less than 40% of the total protein mass (Novo and Esparza, 1981) in contrast to reovirus, where 70% of the protein is in the outer shell (Section 1.3.2.1). Polypeptides VP4.2, VP7.1, VP7.2, VP10 and VP4.3 are all outer shell polypeptides. Of these polypeptides, VP7.1 is the major polypeptide and VP4.2 is the second most abundant. VP3 is present in very small amounts (section 1.3.5.4.2). VP7.2 has been identified as a neutralization-specific antigen (section 6). Only some rotaviruses can grow in tissue culture, and this ability is controlled by the product of gene segment 4 (Greenberg *et al.*, 1981), which is probably VP4.2 or VP4.3 (McCrae and McCorquodale, 1983; Estes *et al.*, 1981).

1.3.2.5 Comparison of rotavirus polypeptides from different serotypes.

Thouless (1979) compared structural polypeptides of several rotavirus serotypes and, while the polypeptide pattern of each serotype was similar, minor migrational differences in

1.3.2.5 (Cont'd.)

all the polypeptides were noted, as seen in Table 5. Cross and Fields (1977) had reported minor differences in all polypeptides of reovirus serotypes 1, 2 and 3, which bears well with the Thouless result. Espejo et al. (1980) compared polypeptides of two human rotavirus types and noted differences in migration of polypeptides VP2, VP3 and the three smallest outer shell polypeptides so, even among different strains of the same serotype, quite a degree of divergence was noted.

This study was extended by Dyal-Smith and Holmes (1981), who compared peptide patterns of the polypeptides from SA-11 and the human Wa strain of rotavirus using single dimension peptide mapping. They found the peptide patterns of all the corresponding inner shell polypeptides as well as of VP10 from the outer shell to be quite similar, but noted marked differences between the two strains in the peptide maps of the outer shell proteins VP3 and VP7 and of the non-structural protein VP5. The polypeptides which differ most in their peptide maps are the ones which are identified as the most type-specific by immune precipitation, in this thesis. The results are consistent with a common ancestral source for the different rotavirus serotypes and evolutionary divergence of selected polypeptides.

1.3.2.6 Reovirus Antigens

Reoviruses, like most other viruses, have been shown to have both group specific and type-specific antigens. The mammalian reoviruses can be divided into 3 serotypes by neutralization and haemagglutination inhibition, but share common antigens in complement fixation, immunodiffusion and immunofluorescence. The avian reoviruses can be divided into 5 serotypes by neutralization, and they also share common antigens. There is no antigenic overlap between avian and mammalian reoviruses.

Reovirus antigens were further characterised by immunoprecipitation. Gaillard and Joklik (1980) immunoprecipitated proteins from reovirus-infected cells with polyspecific antisera and showed that all of them, except for the minor polypeptides $\lambda 3$ and $\mu 2$, were precipitated by antisera raised against purified reovirus. The efficiency of precipitation of each polypeptide was measured as the reciprocal of the antiserum dilution that precipitated half of the protein present. The efficiency of precipitation varied - the outer capsid proteins $\mu 1c$, $\sigma 1$ and $\sigma 3$ and the core protein $\sigma 2$ all reacted well. The non-structural proteins showed the weakest reaction but since the antiserum was raised against purified virus, this was not surprising. The type-specificity of the polypeptides was measured by cross-immune precipitation

1.3.2.6 Reovirus Antigens (Cont'd)

with heterologous antisera, and $\sigma 1$, $\lambda 2$, $\mu 1c$, $\sigma 2$ and $\sigma 3$ all showed a degree of type-specificity. $\sigma 1$ was the most type-specific although not absolutely so - heterologous antisera at a low dilution precipitated $\sigma 1$. Generally the antigens were surprisingly cross-reactive, probably due to some common antigenic determinants. By hybridisation of dsRNA's, serotypes 1 and 3 are related by approximately 40% while serotype 2 is related to serotypes 1 and 3 by less than 10% (Martinson and Lewandowski, 1975) but the antigenic determinants on serotype 2 were as reactive with heterologous antisera as those of the other two serotypes.

Reassortment occurs readily between the RNA segments of three serotypes and, as there are detectable differences in the migration of all the dsRNA's (except L1) between at least two of the serotypes, it is usually possible to determine which gene has reassorted (Ramig et al., 1977). Reassortment experiments have shown that gene S1 codes for the viral haemagglutinin (Weiner et al., 1978) and for the antigen eliciting neutralizing antibody (Weiner and Fields, 1977). The product of gene S1 is the type-specific $\sigma 1$ protein found in the outer shell (McCrae and Joklik, 1978).

1.3.2.6 (Cont'd).

The characterisation of reovirus antigens has been the subject of recent new interest since the production of monoclonal antibodies against reovirus polypeptides (Lee et al., 1981; Burstin et al., 1982). Lee et al. (1981) raised monoclonal antibodies against 7 of the 10 reovirus-specified polypeptides of reovirus type 3. These proteins were $\sigma 2$ and $\lambda 2$ (inner shell capsid proteins), $\mu 1$ and $\mu 1c$ (which are related, but $\mu 1$ is located in the inner shell and $\mu 1c$ in the outer shell), $\sigma 1$ and $\sigma 3$ (outer capsid proteins) and μNS and σNS (non structural proteins). Cleavage products of $\lambda 2$ and μNS , not previously described, were also identified by immune precipitation of infected cell lysates and purified virus using their antisera. Immune precipitation of cell lysates infected with the other two serotypes showed that $\sigma 1$ was undoubtedly the most type specific protein, while $\sigma 3$ was type specific in serotype 2 only. $\mu 1/\mu 1c$ and $\sigma 3$ were thought to be complexed together since most sera directed against any one of these proteins precipitated all three. Hayes et al. (1981) studied the interaction of these monoclonal antibodies with reovirus particles by determining their ability to neutralize, inhibit haemagglutination and aggregate reoviruses. They found that not only did antibodies against $\sigma 1$ ($\alpha \sigma 1$) neutralize viral infectivity and inhibit haemagglutination but that antibodies against $\sigma 3$ and $\lambda 2$ also had these properties.

1.3.2.6 (Cont'd).

However, $\alpha\lambda 2$ was not type specific and $\alpha\sigma 3$ was only partly so. $\sigma 3$ is an outer shell polypeptide, forming outer shell spikes, while $\lambda 2$ is the principle component of the 12 core spikes in the inner shell, but these must pass through the outer shell to be exposed on the reovirus surface. $\lambda\sigma 1$, $\alpha\sigma 3$ and $\alpha\lambda 2$ all precipitated and aggregated viral particles. Some of the $\mu 1/\mu 1c$ and μNS antibodies also aggregated virus particles, suggesting that these also reacted with viral determinants exposed on the viral surface. μNS is not a structural protein but is possibly antigenically related to a structural polypeptide. This relationship is weak as μNS does not immune precipitate structural proteins.

Burstin et al. (1982) isolated a number of monoclonal antibodies directed against $\sigma 1$ of both type 1 and type 3. These monoclonal antibodies, although directed against the same polypeptide, had different biological activities. Of 11 $\alpha\sigma 1$ antibodies from type 3, only two neutralized infectivity efficiently and one neutralized inefficiently. Four other antibody preparations inhibited haemagglutination and one of these also neutralized infectivity. Several antibodies, although they precipitated $\sigma 1$ in cell extracts had neither neutralizing or HI activities. Therefore, four different antigenic sites were defined on the reovirus type 3 $\sigma 1$ molecule - one area was involved in neutralization, another in haemagglutination, another in neither of these,

1.3.2.6 (Cont'd.)

and one monoclonal antibody reacted against an area of the antigen where it could inhibit both neutralization and haemagglutination. Monoclonal antibodies against reovirus type 1 $\sigma 1$ all had neutralizing and HI activities and so did not define different antigenic sites. This could either mean that they were all directed to the same area of $\sigma 1$ or that the arrangement of type 1 $\sigma 1$ differ from that of type 3, although this latter hypothesis seems unlikely. Antigenic sites with different functions on a single virus polypeptide have also been found on the HA of influenza virus (Gerhard et al., 1981; Webster and Laver, 1980) and the major antigen of RNA tumour viruses (Stone and Nowinski, 1980; Massey and Schochetman, 1981) so many antigens appear to have several distinct functions, often located on separate antigenic determinants. The section on rotavirus antigens (1.3.2.7) shows that some of them also have more than one property, but detailed research into the antigenic structure of the polypeptides has not yet been undertaken.

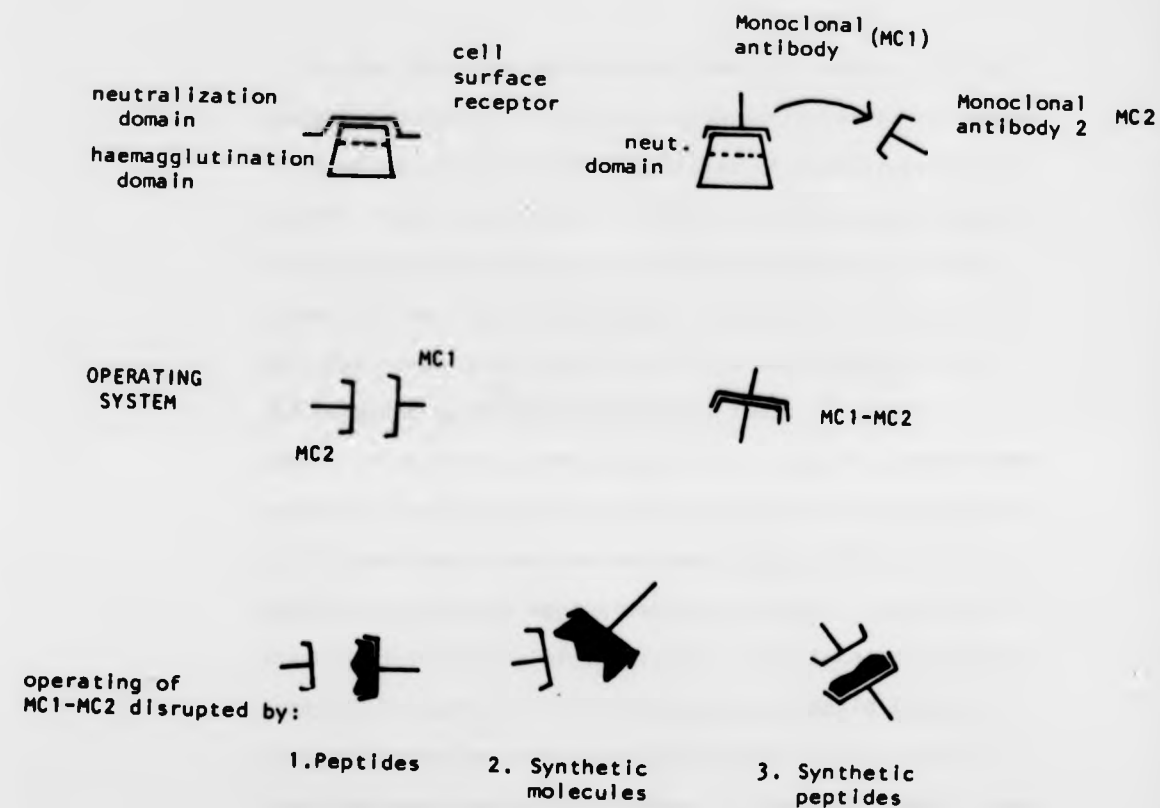
A further development on the study of reovirus antigens has taken place after production of antibodies with activity against the cell surface receptor that binds reovirus (Fields and Greene, 1982). This was achieved by immunizing mice with the monoclonal antibody (MC-1) known to interact with the neutralizing region of $\sigma 1$, and screening for

1.3.2.6 (Cont'd).

monoclonal antibodies (MC-2) with binding activity to MC-1 (fig.8). The assumption that the binding region of MC-1 mimicked the cell receptor was correct as MC-2 was able to block cell receptors against virus and to mimic viral binding to the receptors. The antibody also interacted with lymphocytes in the same way as virus and activated subsets of T cells. This antibody is important for two reasons - firstly, it could be used to induce immunity without exposure to virus or to confer passive protection by blocking the cell receptors, and it has been exploited as a model system to identify which tryptic peptides of the haemagglutinin or other synthetic molecules or peptides block the binding of MC-1 to MC-2. Any molecules which block this binding would be potentially useful as a vaccine (fig.8).

Fig.8 Definition of an operational analogue of virus-cell receptor interactions.

In the reovirus system, the neutralization domain of haemagglutinin interacts with cell surface receptors and the antigen-binding site of the monoclonal antibody (MC1) directed against that domain. A second monoclonal antibody (MC2) made against the antigen-binding site of the first interacts with the cell surface receptor for the neutralization domain and also interacts with MC1. The MC1-MC2 interaction appears to be the analogue of the virus-cell receptor interaction and constitutes the operating system for studying proteins which can be used as potential immunogens. Only those proteins (or synthetic molecules) that interfere with MC1-MC2 binding are likely to be useful vaccines (reproduced from Fields and Greene, 1982).



1.3.2.7 Rotavirus Antigens

Section 1.2.6 described evidence for the presence of group and type specific antigens in rotavirus particles, and for the existence of several serotypes.

On the basis of IEM observations that double shelled human rotaviruses reacted only with antiserum to the human virus while single shelled particles of rotaviruses from several animals cross-reacted with all convalescent sera from calves, pigs and sheep tested, Woode et al. (1976) suggested that rotavirus group antigen(s) were located on the inner capsid of the virus. This was substantiated and extended by Bridger (1978) who found, on testing several rotavirus serotypes and their specific convalescent antisera, that single shelled particles were agglutinated by all positive rotavirus antisera, while double shelled particles were only agglutinable by antisera that neutralized the infectivity of the virus. This located the type specific antigen in the rotavirus outer capsid layer and suggested that the group specific antigen is an inner shell polypeptide which is masked by the outer capsid layer, at least to antibodies that agglutinate rotavirus particles. However, Bastardo et al. (1981) found that monospecific antisera directed against denatured outer shell polypeptides VP4.2, VP7 and VP10 of SA-11 agglutinated complete particles of both SA-11 and calf rotavirus, so were not type-specific. The antiserum against inner shell polypeptide VP6 agglutinated single-

1.3.2.7 (Cont'd.)

shelled particles of SA-11 and calf rotavirus. Antiserum against VP6 was the only one which reacted as efficiently with calf rotavirus as with SA-11 in CF, so some type-specificity of outer shell proteins was demonstrated by this technique.

The outer shell of rotavirus is also shown to be type-specific by haemagglutination, as studied by Inaba *et al.* (1977) and Fauvel *et al.* (1978). Haemagglutination is only observed with double-shelled particles and only inhibited by antisera that neutralize the virus. Experiments with monospecific antisera suggest that the haemagglutinin and neutralization antigen may reside on the same polypeptide, as in reovirus (Weiner and Fields, 1977; Weiner *et al.*, 1978; Bastardo *et al.*, 1981; Matsuno and Inouye, 1983) but results of genetic experiments do not conform to this theory as the neutralization-specific antigen was assigned to gene segment 8/9 while the haemagglutinin was assigned to gene segment 4 which codes for VP3 (Kalica *et al.*, 1981; 1983). Neutralization assays using the monospecific antisera identified three different antigens involved in type-specific neutralization - VP6, one of the VP7 group, and VP10, the most important of which appeared to be the VP7 polypeptide (Bastardo *et al.*, 1981; Matsuno and Inouye, 1983). Only antiserum raised against VP7 inhibited haemagglutination. Most groups

1.3.2.7 (Cont'd.)

recognise the existence of two VP7 polypeptides in virus particles, which we call VP7.1 and VP7.2 (see fig.12), but these were not differentiated by the experiments of Bastardo et al. (1981). Chapter 7 describes experiments to identify the antigen involved in neutralization. Monoclonal antibodies which precipitate VP3 and VP7 from infected cells were both found to neutralize rotavirus infectivity (Greenberg et al., 1983b), so VP3/VP4.2 is another antigen which has been implicated in neutralization. Surprisingly, monoclonal antisera against VP3 were type-specific in neutralization, while those against VP7 were not. Both types of monoclonal antisera inhibited haemagglutination and all but one of the α VP7 sera were type-specific, so α VP7 must have some involvement in haemagglutination inhibition despite the fact that genetic experiments have assigned this function to VP3. The authors suggested α VP7 could inhibit haemagglutination by steric hinderance.

In other virus systems, more than one type-specific and group-specific antigen have often been found. In these cases, most virus polypeptides have been found to elicit the synthesis of specific antibody, as most of them can be precipitated by these antisera (Gaillard and Joklik, 1980; Harris et al., 1981; Yeo et al., 1981; Bernstein and Hruska, 1981), and several were shown to be precipitated by heterologous antisera and were therefore group-specific. This thesis presents evidence for the existence of more than one group-specific antigen in calf rotavirus (section 5).

1.3.2.7 (Cont'd.)

Rotaviruses are classified either by neutralization, ELISA or IAHA. Experiments with reassortant viruses described in section 1.3.5.2 have shown that the ELISA subgroup is determined by the product of gene segment 6 (VP6), while type-specific neutralization is a product of gene segment 8 or 9 (probably coding for VP7) (Kalica *et al.*, 1981). VP6 appears to have both type-specific determinants in neutralization, and subgroup-specific determinants, which are common to many serotypes, in ELISA (Bastardo *et al.*, 1981; Kalica *et al.*, 1981) (Chapter 6). Use of monoclonal antisera against VP6 have identified that VP6 has both group-specific and subgroup-specific determinants by the ELISA test (Greenberg *et al.*, 1983a). Curiously, each ELISA subgroup is also associated with a major difference in the migration of RNA segments 10 and 11 (Kalica *et al.*, 1981b) although the products of these gene segments are not subgroup-specific determinants (Kalica *et al.*, 1981).

Thus it appears that there are several rotavirus antigens involved in group-specific reactions, subgroup-specific reactions and type-specific (neutralization) reactions. The situation is still somewhat confused at present and clearly more research is necessary to understand the different antigenic properties of each rotavirus polypeptide.

1.3.3 RNA polymerases

1.3.3.1 The RNA polymerase of reoviruses.

Reoviruses have their own virus-associated RNA polymerase, which confers several advantages to the virus as summarised by Bishop (1977). A viral polymerase can be used solely for transcription and replication of the viral genome and this avoids competition for cellular enzymes and allows efficient repetitive transcription of the viral mRNA - in the case of the reoviruses, transcription in vitro often yields more than the virus' own weight of mRNA. The polymerase avoids the necessity for any protein synthesis between infection and transcription, and enables the virus to evolve its own mechanisms for transcriptional control and its own initiation/termination sites on the viral genome. This aspect of reovirus is discussed in section 1.3.1.3. The viral genome does not have to leave the virus particle and can therefore be protected from cellular ribonucleases.

The reovirus RNA polymerase was first described in 1968 by Shatkin and Sipe and by Borsa and Graham, only a year after the discovery of the first virus-coded, virus associated RNA polymerase, the vaccinia virus polymerase (Kates and McAuslan, 1967). Since then a polymerase has been identified in all of the reoviruses studied. Most of the work has been done on reovirus, so this will be the subject of discussion.

1.3.3.1 (Cont'd).

The reovirus dsRNA polymerase is often termed a 'transcriptase' as its sole function is to transcribe mRNA. It is located in the viral cores and transcribes all 10 dsRNA species into mRNA. Four other virus associated enzymes also contribute to the transcription and modification of mRNA (1) nucleoside triphosphate (NTP) phosphohydrolase, which hydrolyses NTP's to diphosphates and removes the 5' terminal phosphate from nascent message, (2) terminal guanyl transferase and (3 and 4) enzymes that methylate both the cap 5 and the ribose of the original 5' terminal residue (Kapuler et al., 1970; Borsa et al., 1970; Furulich et al., 1975 and Shatkin, 1974). These enzymes will be discussed in more detail later. A virion associated pyrophosphate exchange enzyme that catalyses the reaction $[\beta, \gamma^{32}\text{P}]\text{GTP} + \text{PP}_i = \text{GTP} + {}^{32}\text{PP}_i$ also exists (Waschman et al., 1970). All of these enzymes are only expressed when the viral outer capsid shell is completely or partially removed. (Shatkin and Sipe, 1968; Borsa and Graham, 1968). Chymotrypsin at 50 $\mu\text{g/ml}$ or heat shock produces a subviral particle capable of showing RNA polymerase activity (Joklik, 1972). Activation is achieved by removal of $\sigma 3$ and some, but not all, of the μlc polypeptide (Joklik, 1972) although reovirus cores, which also transcribe mRNA, have lost all the outer shell proteins. The reovirus proteins are listed in table 3. This activation possibly

1.3.3.1 (Cont'd)

allows access of precursor nucleotides into the viral core. Addition of σ_3 to activated particles repressed the transcriptase, so it can be suggested that σ_3 blocks specific 'ports', that allow entry of nucleotides and release of transcribed messages in and out of the viral core (Astell et al., 1972). An electron microscopic analysis of actively transcribing reovirus core particles by Gillies et al. (1971) showed them extruding up to 10 messenger RNA molecules, each one from a different site, so these sites could be specific ports for the exit of messages. It is now thought that the messages extrude from the viral spikes which are believed to be hollow (Joklik, 1981). These spikes are composed of λ_2 clusters which could be open in cores and closed in complete particles, and the enzyme could be activated by that or other configurational changes caused by removal of outer shell polypeptides (Joklik, 1981).

1.3.3.1.2 Polypeptides involved in transcription.

The polypeptides that are involved in transcription or capping have not been identified. Disruption of the cores causes all polymerase activity to be lost, so the polymerase enzyme(s) cannot be purified in an active state as has been done with vesicular stomatitis virus (Emerson and Wagner, 1972, 1973; Emerson and Yu, 1975). The fact that disruption of cores causes enzyme inactivation

1.3.3.1.2 (Cont'd).

suggests that the enzymes are components of the core shell. The catalytic sites are probably located on the inside of the core shell as cores transcribe exogenous dsRNA very poorly. Joklik (1981) suggested that the ds templates could move past the enzymes, and the mRNA's could be simultaneously fed out of the spike bases or ports. Morgan and Kingsbury (1980) labelled putative polymerase enzymes by allowing transcription of reovirus in the presence of radiolabelled pyridoxal phosphate, which is thought to react with the enzymes' active site, and they found that $\lambda 1$ and $\lambda 2$ became labelled. $\lambda 2$ is the spike protein and $\lambda 1$ is positioned beside it in cores (section 1.3.2). While this is not proof of these proteins being involved in transcription, it fits in with the model that the spike bases are actively involved. $\lambda 1$ and/or $\lambda 2$ could be polymerase and $\lambda 2$ also involved in capping the nascent message before extrusion. The other core proteins $\lambda 3$, $\mu 1$ and $\mu 2$ have not been assigned any function, but could also be involved in transcription. A similarity of the size and molar ratios of $\lambda 1$, $\lambda 1$ and $\sigma 2$ to the E.coli RNA polymerase β, β' and α subunits has been noted.

1.3.3.1.3 The reovirus transcription reaction.

The transcription reaction of reovirus and the basic characteristics of the transcriptase enzyme have been studied extensively. The reaction requires all four nucleoside triphosphates and Mg^{++} ions at an optimum of 13mM. The pH optimum is pH 8.0, and the reaction does not require the presence of a reducing agent. The polymerase is not inhibited by a high concentration of actinomycin D, an inhibitor of DNA to RNA polymerase, but is inhibited by 0.01M phosphate. The optimum temperature is unusually high at 50°C (Shatkin and Sipe, 1968b) Borsa and Graham, 1968; Skehel and Joklik, 1969). Most other viruses, including rhabdoviruses, orthomyxoviruses and paramyxoviruses, as well as some other reovirus strains, e.g. cytoplasmic polyhedrosis virus, blue tongue and wound tumour viruses, have optimum transcription temperatures of 25-30°C (Bishop, 1977). The reovirus enzyme activity is evidently more stable than most enzymes and transcription can be sustained for 48h at 37°C provided enough NTP's and an ATP (energy) generating system (phosphoenolpyruvate + pyruvate kinase) are supplied to sustain the reaction (Skehel and Joklik, 1969). In vitro only 'positive polarity' (mRNA sense) molecules are produced (Skehel and Joklik, 1969). The first plus strand to be produced is a genuine transcript, i.e., the original ds template is not separated during transcription (Skehel and Joklik, 1969), hence transcription is asymmetric and conservative. It is not known how transcription occurs

1.3.3.1.3 (Cont'd).

on duplex RNA without displacement of the parental plus strand, but probably involves a local, transitory displacement which is reversed as an area of template is transcribed.

1.3.3.1.4 Characterisation of the products of transcription by reovirus.

The transcripts synthesised in vitro are the same length as the double stranded RNA genome (Skehel and Joklik, 1969) and the same length as mRNA extracted from infected cells (Hay and Joklik, 1971). All 10 genome segments are transcribed into full length messages, and the mRNA's are therefore sized into three size classes - large (ℓ_1 , ℓ_2 , ℓ_3) medium (m_1 , m_2 , m_3) and small (s_1 , s_2 , s_3 , s_4).

The rates of transcription, under optimal conditions, are the same for all the genes and therefore more s transcripts are produced than m transcripts, and more m transcripts produced than ℓ transcripts. The frequency of initiation is thus inversely proportional to genome size, so it is unlikely that the 10 genes are transcribed as a linked complex. The total rate of RNA synthesis has been estimated to incorporate 10-60 nucleotides/second with complete molecules of the largest species present within 8 min. (Skehel and Joklik, 1969; Bannerjee and Shatkin, 1970). The relative rates of transcription in vivo are

1.3.3.1.4 (Cont'd).

completely different. Early in infection, or in cycloheximide treated cells, Watanabe et al. (1968) and Shatkin and La Flandra (1972) observed only (23)m3, s3 and s4. Later in infection all 10 mRNA species are found but they are neither in equimolar or in equimass proportions (Zweerink and Joklik, 1970). The approximate transcription frequencies are shown in table 7. Under less than optimal conditions in vitro, the relative rates of transcription of the various RNA species synthesised are not the same. Decrease in magnesium concentration causes a decrease in synthesis of 2 species, and if only trace amounts of ATP are present, only some sRNA species are made (Joklik, 1974; Nichols et al., 1972). This is possibly how transcription is regulated in vivo.

Initiation of transcription appears to be a complex and sensitive process and it seems that transcription aborts in more than 50% of cases when transcripts are still less than five residues long (Yamakawa et al., 1981). The 5'-terminated oligonucleotides present in reovirus particles (section 1.3.1.2) are thought to be products of abortive transcription. These sequences may be abortive transcripts from the standard transcription process, or may be produced late in transcription if the elongation process, but not initiation, is inhibited giving a

Table 7

Transcription frequencies of each of the
reovirus genes (from Joklik, 1981).

<u>Gene</u>	<u>Transcription frequency</u>
L1	0.05
L2	0.05
L3	0.05
M1	0.15
M2	0.3
M3	0.5
S1	0.5
S2	0.5
S3	1.0
S4	1.0

1.3.3.1.4 (Cont'd).

transitory abortion rate of 100%. The oligoadenylates which are also found in reovirus particles may be produced at the end of the transcription cycle when the transcriptase is being inactivated. In these circumstances, the K_m for ATP could be higher than that for other NTP's and allow template-independent polymerisation of ATP. Production of the oligoadenylates has indeed been shown to occur during the later stages of morphogenesis (Silverstein et al., 1974; Stoltzfus et al., 1974). Late temperature sensitive mutants that assemble non-infectious particles do not synthesise oligoadenylates at non-permissive temperatures (Johnston et al., 1976). Oligo A polymerase activity is manifest in intact virions and has a preference for Mg^{++} ions (Silverstein et al., 1974). Silverstein et al. (1976) suggested that this enzyme is an alternative activity of the virion bound transcriptase and that the constraints of the outer capsid proteins inhibit the transcriptase activity but allow oligoadenylate synthetase activity. The existence of a separate enzyme for this latter function has not been ruled out.

The full length messages that are produced lack a Poly A sequence at their 3' end that is common to many mammalian mRNA's (Stoltzfus et al., 1973). 3' poly A is

1.3.3.1.4 (Cont'd).

thought to have an important function in mRNA stability (Revel and Groner, 1978), but reovirus mRNA appears to be as stable as other mRNA's. The mRNA molecules become capped and methylated at their 5' ends if the methyl donor S-adenosyl methionine (SAM) is present (Shatkin, 1974) and these 5' caps were shown to have some role in increasing the efficiency of translation (Both *et al.*, 1975b) Muthukrishnan *et al.*, 1975).

SAM is an important constituent in CPV transcription reactions and stimulates the transcription rate by up to 70-fold (Furuichi, 1974), since CPV transcription is coupled with methylation of the product RNA.

Analysis of the 5' sequences of CPV dsRNA and mRNA transcribed *in vitro* revealed that the 5' sequences of the minus strand were ppGpGpCp and those of the plus strand and of products of *in vitro* transcription were m⁷GpppAmpGpUp.... [m⁷G is 7-methylguanosine and Amp is 2'-O-methyladenylic acid(ribose methylated)] while the 3' sequences were pXpCpU_{OH} and pXpCpC_{OH} respectively (Miura *et al.*, 1974; Furuichi and Miura, 1975). These sequences are all summarised in fig. 9. This implied that during transcription an inverted guanosine nucleotide (cap) is added to the 5' end and the guanosine is then methylated as well as the ribose of the next nucleotide. Reovirus cores synthesise product RNA species equally effectively in the presence or absence of SAM (Furuichi

FIGURE 9 (From Bishop 1977).

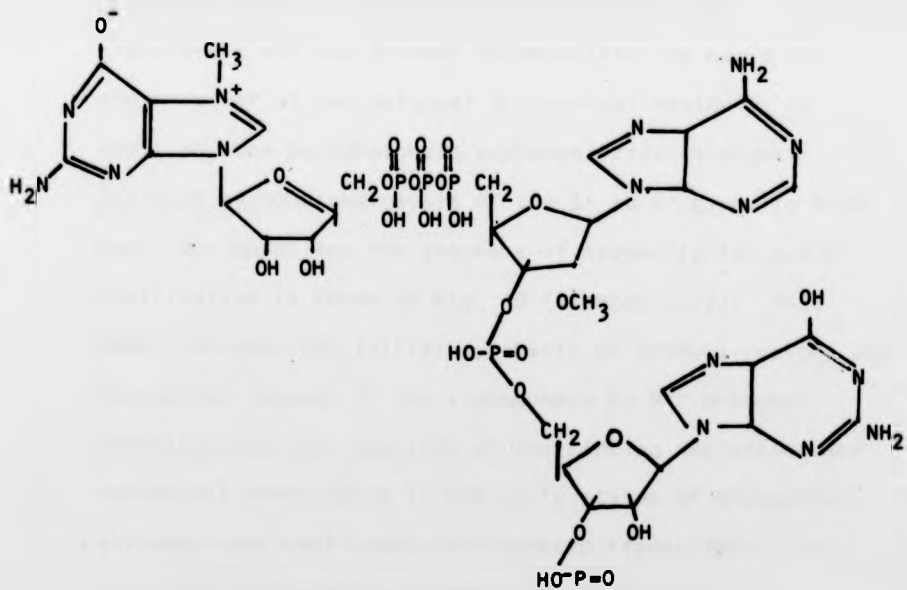
The 3' and 5'-nucleoside sequences of CPV and reovirus

CPV $m^7GpppAmpGpUp$ $pXpCpC_{OH}$ + strand
 $H_2OUpCpXp$ $pCpGpGpp$ - strand

 mRNA $m^7GpppAmpGpUp$

Reovirus $m^7GpppGmpCpUp$ $pApUpC_{OH}$ + strand
 $H_2OCpGpAp$ $pUpApGpp$ - strand

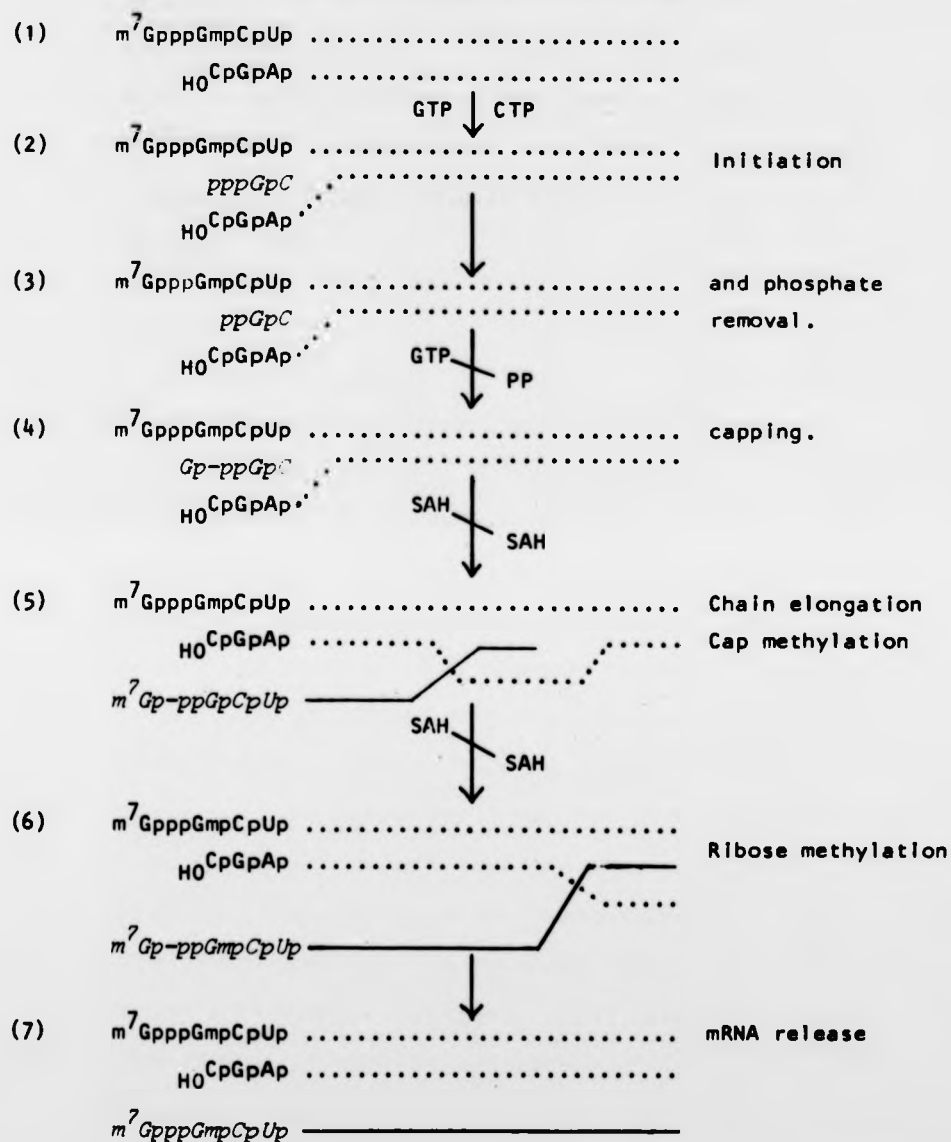
 mRNA $m^7GpppGmpCpUp$



1.3.3.1.4 (Cont'd).

et al., 1975) but when SAM is added, the sequence of the product 5' ends changes from ppGpCp... to m⁷G^{5'}ppp^{5'}GmpCp (Bannerjee et al., 1971; Furuichi et al., 1975; Levin et al., 1970). The 3' and 5' sequences of reovirus dsRNA and mRNA are shown in fig.9. Methylation was shown not to involve an m⁷GTP and therefore occurred in situ (Bishop, 1977). The methylation is specifically blocked by S-adenosyl homocysteine (Both et al., 1975). The enzymes responsible for methylation and capping in reovirus virions have not been identified, but involve the four activities: NTP phosphohydrolase, terminal guanylyl transferase and the enzymes to methylate the cap G and the ribose of the original 5' terminal residue. In addition, the pyrophosphate exchange activity might activate pyrophosphorolysis of the 5' to 5' guanylic acid cap. One model for the sequence of transcription and 5' modification is shown in fig. 10 (Bishop, 1977). This model includes the initial synthesis of product pppGpCp and subsequent removal of the γ-phosphate by NTP phosphohydrolase and then addition of the capping nucleotides and subsequent methylation in the early stages of elongation, although this could occur on released transcripts. The following observations suggest that capping and methylation are virus coded functions (i) methylation and transcription in CPV are coupled (ii) Reovirus, CPV and wound tumour virus can cap and methylate transcripts

FIG.10 Model for Sequence of Transcription and 5' Modification
for Reovirus mRNA Transcripts



1.3.3.1.4 (Cont'd).

in vitro (iii) The capping and methylating activities are substrate specific - $^{32}\text{ppGpCp...}$ dinucleotides given to reovirus cores can be capped and methylated to $\text{Gp}^{32}\text{ppGpCp...}$ and GpppGp... to $\text{m}^7\text{GpppG...}$, but GpppA can not be methylated (e.g. to $\text{m}^2\text{GpppA...}$ - the VSV cap) (Bishop, 1977).

1.3.3.2 Rotavirus RNA polymerase

A polymerase enzyme was identified in purified calf rotavirus in 1977 (Cohen, 1977), and was associated with 'denser' virus particles which were derived from infectious virus by treatment with 1mM EDTA or heat shock. EDTA treatment removed the viral outer shell by chelating Ca^{++} ions which bound the outer shell polypeptides (Cohen et al., 1979), so the 'denser' particles were single shelled, non-infectious rotavirus. The rotavirus polymerase was similar to its reovirus counterpart by its association with single shelled particles and optimum activity at 45-50°C, in the presence of 10mM Mg^{++} and at pH 8.0. The polymerase product was sensitive to pancreatic RNAase but was rendered RNAase resistant after annealing with calf rotavirus dsRNA, suggesting that the product was ssRNA as in the case of reovirus. Hruska et al., (1978) later identified an RNA polymerase associated with human rotavirus isolated from the stools of patients with diarrhoea. The enzyme was associated with the denser virus band of 138g/cm³ in CsCl gradients but not with the lighter band of 1.36 g/cm³. Recently, Spencer and Arias (1981) studied the properties of the human rotavirus RNA polymerase activated by heat shock. They identified a requirement for monovalent cations such as K^{+} or Na^{+} (100mM) and showed that the reaction was not inhibited by phosphate ions, actinomycin-D, α -amanitin or rifampicin. They also showed that a hydrolysable form of ATP, but not of CTP, GTP

1.3.3.2 (Cont'd).

or UTP, was necessarily for the polymerase activity, suggesting that ATP is also used as an energy source.

1.3.3.2.2 Characterisation of the products of rotavirus transcription

Cohen and Dobos (1979) and Mason et al.(1980) both characterised the transcription products by agarose (and agarose-acrylamide) gel electrophoresis and found 8 separate bands of ssRNA, the largest of which comigrated with 23S ribosomal RNA. The molecular weight range of these ssRNA's was 2×10^5 to 1.2×10^6 . Hybridisation of transcription products to parental dsRNA, and polyacrylamide gel analysis of the transcripts was attempted by Cohen and Dobos (1979) and also by Bernstein and Hruska (1981b). Both found that, while all dsRNA species hybridised to transcription products, the high molecular weight transcripts were present in far lower quantities than the lower molecular weight transcripts, i.e. even less than would be expected on the basis of production of equimass amounts of each mRNA. This could be partly due to the presence of ribonucleases in the transcription mix (possibly associated with virus particles), but even in the presence of ribonuclease, fragments of RNA should still hybridise to genomic dsRNA and the hybrids should be visualised on gels. Mason et al.(1980), using simian rotavirus SA-11 showed that shorter transcription

1.3.3.2.2. (Cont'd).

Incubation times (2h instead of 8h or 18h) allowed production of more high molecular weight transcripts and concluded that ribonucleases were present in the transcription mix. Using mRNA from 2h incubation, Mason et al. (1980) found that the transcripts had an average sedimentation velocity of 12S on sucrose gradient sedimentation. The products did not fall into distinct size classes, as in the case of reovirus mRNA, but formed a single broad peak. Bernstein and Hruska (1981) further analysed the transcripts by showing that they were of one polarity and identical in size to parental dsRNA segments. Like reovirus, the transcripts were shown to lack 3' polyadenylate sequences. The product was shown by CsCl gradient fractionation to be released from the virion, leaving the virion with intact polymerase activity.

1.3.4 Reovirus multiplication

As the reovirus multiplication cycle is well understood, it is useful to describe it as a model for rotavirus replication. The current knowledge on this subject will be outlined afterwards in section 1.3.5.

1.3.4.1 Early stages of replication: adsorption and uptake

Most of the work on the reovirus growth cycle has used the mammalian serotype 3 in mouse L cells. Reovirus particles adsorb to specific cell surface receptors, and competition experiments have shown that these receptors are the same for all three serotypes (Lee et al., 1981b). The reovirus protein responsible for adsorption is $\sigma 1$ - it is a minor polypeptide present in pairs in 12 locations on the particle surface. Free $\sigma 1$ can also adsorb to cells (Lee et al., 1981b). The adsorption rate of reovirus is similar to that of vaccinia virus with 65% of virus adsorbed to cells within 30 min under standard conditions (Joklik, 1972) and the adsorption rate constant has been calculated to be about 2.5×10^{-9} cm³/min/cell.

Reovirus particles are taken into cells by phagocytic vacuoles, which subsequently fuse with lysosomes (Dales et al., 1965; Silverstein and Dales, 1968). This process is very efficient and most of the virions adsorbed are found in secondary lysosomal vesicles within 30 min of adsorption.

1.3.4.1.2 Uncoating

After adsorption and uptake, reoviruses are degraded to subviral particles (SVP's) in secondary lysosomal vesicles within 1 hour after adsorption (Silverstein et al., 1970; 1972). The conversion to SVP's is apparently effected by lysosomal enzymes and can also be achieved by chymotrypsin under appropriate conditions. The SVP's are infectious and have full transcriptase activity while the genome RNA is protected from ribonucleases. This partial uncoating is unusual as most animal viruses uncoat more completely to expose their genomes or ribonucleoprotein complexes (Shatkin et al., 1977). The conversion of virus to SVP involves removal of about 50% of the outer capsid protein: proteins $\sigma 1$ and $\sigma 3$ are removed completely and a 12,000 dalton fragment is cleaved from $\mu 1c$ leaving a 60,000 dalton protein called σ (Silverstein et al., 1972). The SVP's produced are smaller, about 0.02 g/ml denser and contain all the virus dsRNA (Joklik, 1974).

The SVP's are then transferred, during the next 2-4h to dense perinuclear inclusions associated with mitotic spindles that develop into "viral factories" and are the sites of viral RNA and protein synthesis and morphogenesis (Dales, 1963; Dales et al., 1965; Anderson and Doane, 1966; Fields et al., 1971).

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The SVP's are then transferred, during the next 2-4h to dense perinuclear inclusions associated with mitotic spindles that develop into "viral factories" and are the sites of viral RNA and protein synthesis and morphogenesis (Dales, 1963; Dales et al., 1965; Anderson and Doane, 1966; Fields et al., 1971).

1.3.4.2 Reovirus transcription in vivo

Transcription in infected cells is undertaken by the SVP's, which synthesise single stranded (ss) mRNA of one polarity. Each ssRNA segment is a full length copy of its ds parent RNA. The transcription product is extruded from SVP's and acts as mRNA in translation as well as template for ss to dsRNA synthesis. All 10 mRNA's are probably transcribed together, involving multiple transcription sites as found in vitro (see section 1.3.3.1.2).

Two phases of mRNA synthesis have been identified in a one-step multiplication cycle (Bellamy and Joklik, 1967; Watanabe et al., 1967). The lag period between uncoating and transcription lasts more than an hour, probably allowing for release of SVP's from secondary lysosomal vesicles. mRNA is not produced in lysosomes, either because mRNA's are quickly degraded within lysosomes or because nucleoside triphosphates (NTP's) are not found there. The first stage of transcription is a slow, early phase where mRNA is transcribed from the parental genome after uncoating, and this stage is independent of protein synthesis. The mRNA's produced are capped and methylated and are active in in vitro translation systems (Skup and Millward, 1980). The conservative nature of reovirus replication means that this early mRNA is the sole carrier of genetic

1.3.4.2 (Cont'd)

Information from parent to progeny virions and some or all of the mRNA's produced in the early stage become packaged in immature particles and copied into dsRNA molecules. The later (second) phase of mRNA synthesis takes place after synthesis of progeny dsRNA and involves the transcription of progeny dsRNA molecules within immature particles. This later transcription is faster and the amount of mRNA produced greatly exceeds that of early mRNA. Studies using ts mutants negative for dsRNA synthesis or cycloheximide to block protein synthesis and hence later transcription, suggest that 80-95% of transcription in infected cells occurs in the late phase (Ito and Joklik, 1972; Watanabe *et al.*, 1967). The mRNA produced by progeny particles has been found to be uncapped, the capping enzymes being latent in these immature particles (Skup and Millward, 1980).

1.3.4.2.2 Control of transcription

The relative rate of transcription of each of the mRNA species has been determined by hybridisation of newly formed transcripts to genome RNA by several groups, who provide conflicting data. Zweerink and Joklik (1970) measured the rate of transcription of each mRNA species from 2h p.i., when mRNA was first detectable until the end of the growth cycle. They observed that each mRNA

1.3.4.2.2 (Cont'd).

species was produced at a constant rate throughout the infection cycle, but that these rates were different from those seen in vitro, as summarised in table 7. Production of the larger mRNA species was observed to be relatively slower than in vitro, possibly due to the presence of less than optimal concentrations of Mg^{++} and NTP's in the cells as the concentrations of these components affect the relative transcription rates of each mRNA in vitro (see section 1.3.3.1.3). Control of Mg^{++} and NTP concentration inside cells is thought to be a possible method of control of the rate of transcription by reoviruses (Bishop, 1977). The higher relative frequency of transcription of small mRNA's is also thought to be partly due to the observed sequential synthesis of dsRNA - small species are formed first followed by m and ℓ species, which could leave a steady state situation where there are more smaller dsRNA templates for transcription (Zweerink, 1974).

Watanabe et al. (1968) also studied the relative transcription rate of each mRNA throughout the cycle, but produced different results. They found that during early transcription, and during transcription in the presence of cycloheximide, only four RNA species were made - s4, s3, m3 and ℓ 1, while late transcription

1.3.4.2.2. (Cont'd.)

allowed production of all 10 mRNA species. Shatkin and La Fiandra (1972) and Lau et al. (1975) also observed production of the same four mRNA species on infection of cells in the presence of cycloheximide. Spandidos and Graham (1976) observed production of the same four mRNA species during non permissive infection of mammalian L cells with an avian reovirus. The data therefore suggest that very early in infection there is some regulation of transcription of reovirus genes. The regulation is probably by a host cell factor, the function of which is neutralised by the gene products of one of the four genes transcribed, as SVP's isolated from infected cells or produced by chymotrypsin digestion produce all 10 species of mRNA in vitro (Levin et al., 1970; Shatkin and La Fiandra, 1972); and ts mutants which produce less than 1% of the normal amount of dsRNA can still transcribe all 10 species of mRNA (Cross and Fields, 1972; Ito and Joklik, 1972). SVP's appear to be impermeable to even small proteins such as ribonucleases, so the mechanism of regulation of transcription within SVP's by an outside factor is at present a mystery.

1.3.4.2.3 Production of oligonucleotides

During later stages of morphogenesis, transcription appears to be aborted unusually frequently and this produces large quantities of 5' G-terminated oligonucleotides (as described in Section 1.3.3.1.4), which all have sequences similar to the 5' sequences of reovirus '+' strands (Bellamy et al., 1972). Poly A polymerase activity also occurs at later stages of the multiplication cycle in vivo (Silverstein et al., 1974). Oligonucleotides and oligoadenylates become sealed into the reovirus particles which become impermeable to NTP's and oligonucleotides once they have acquired $\sigma 3$ (the last stage in maturation). About 25% of the RNA in mature reovirus particles are these oligonucleotides (see Section 1.3.1.2), which have no known function.

1.3.4.3 Translation of reovirus mRNA in vivo

Zweerink and Joklik (1970) analysed protein synthesis in reovirus infected L cells at various stages after infection. During the early period of transcription reovirus protein synthesis was shown to proceed very slowly, but after onset of late mRNA production the virus specified protein synthesis soon replaced that of the host. Using polyacrylamide gel analysis this group identified production of all the structural proteins described by Smith et al. (1969), as well as two other proteins which they called non-structural.

1.3.4.3. Translation of reovirus mRNA in vivo (Cont'd.)

These were μ_0 and σ_4 (now called μ NS and σ NS). Early protein synthesis, during which only a small proportion of the proteins synthesised in cells were viral, was analysed by using actinomycin D and profile subtraction or immune precipitation, but later stages of protein synthesis could be analysed directly. The same polypeptide pattern was found at all stages in infection.

Throughout the protein synthesis period, approximately 67% of the viral mRNA was found to be associated with polysomes, and each mRNA species was bound to ribosomes in approximately the same relative proportion as that in which it was transcribed (Ward et al., 1972). This suggested that all the mRNA's have the same relative affinity for ribosomes. In some cases the mRNA's were found linked together - on heavy ribosomes some of the mRNA was found to be linked in the form of ribonucleoprotein complexes containing capsid polypeptides of all 3 size classes (Ward and Shatkin, 1972). These complexes were thought to have some role in the regulation of protein synthesis.

Both et al. (1975) improved the gel resolution and found two more reovirus-specified proteins in

1.3.4.3 (Cont'd.)

infected cells, bringing the total to eleven. All of these polypeptides were also found after in vitro translation except μlc ($\mu 2$ in old nomenclature) which was present in large quantities in the cytoplasm. Pulse-chase analysis showed that μlc was not a primary gene product since incorporation of radiolabel into μlc increased with labelling time, while the incorporation into all other proteins was independent of time. Also, as the amount of μlc increased the amount of μl concomitantly decreased. Most of the μl produced was converted into μlc , by removal of an 8K MW fragment from the amino terminus of μl (Pett et al., 1973). The enzyme responsible for this cleavage did not operate in any in vitro translation system, and has not been identified. Recently, other cleavage products of reovirus primary gene products have been identified by immune precipitation of infected cells with monoclonal antibody (Lee et al., 1981b). These were cleavage products of μNS (μNSc) and $\lambda 2$ ($\lambda 2\text{c}$) respectively. About 50% of μNS and 10% of $\lambda 2$ were shown to be cleaved but the functions of the two cleavage products were not determined.

Three extra polypeptides (called X, Y, Z) are detected in cells infected at 37°C but not at 31°C. These are not capsid proteins and are not essential

1.3.4.3. (Cont'd).

for growth as virus yields are higher at 31°C than 37°C. X, Y and Z are therefore called 'non-essential non-capsid polypeptides', and are probably breakdown products of other polypeptides caused by leakage of proteolytic enzymes from lysosomes.

All the capsid polypeptides found in infected cells are very insoluble, and most are found as particulate structures or aggregates that range in size and complexity from simple complexes of $\mu 2$ and $\sigma 3$ to full or empty capsids, and these are possibly important intermediates in morphogenesis.

1.3.4.3.2. Regulation of translation in infected cells

Reovirus translation in vivo is controlled at several levels. The frequency of translation of each of the 10 mRNA's varies widely as shown in Table 8. The same relative efficiencies of translation apply throughout the growth cycle as well as in vitro, implying that the most important, if not the only, mechanism for regulating the translation frequencies of the 10 species is the sequence content. Sequencing of reovirus mRNA's is of great interest especially as identification of sequences specifying efficiency of translation would be relevant to

TABLE 8 Approximate relative frequencies of
transcription and translation of the
10 reovirus genes.

Gene	Transcription frequency	Translation frequency	Translation frequency/transcription frequency
L1	0.05	0.03	0.6
L2	0.05	0.15	3
L3	0.05	0.1	2
M1	0.15	0.03	0.2
M2	0.3	1.0	3.3
M3	0.5	0.5	1
S1	0.5	0.05	0.1
S2	0.5	0.2	0.4
S3	1.0	0.3	0.3
S4	1.0	0.7	0.7

1.3.4.3.2. (Cont'd.)

translation of cellular mRNA's. Current knowledge of reovirus sequences is summarised in section 1.3.1.3.

Control is also mediated by the reovirus 5' cap. 5'caps of many eucaryotic mRNA's have been considered important for their translation, capped mRNA's being translated more efficiently in vitro than uncapped mRNA's (Shatkin, 1976), although naturally uncapped mRNA's (e.g. picornavirus mRNA's) are still translated efficiently in cell free systems. More specifically, the 5' cap seems to be an important recognition mechanism for initial ribosome binding and is also important for the general stability of mRNA molecules in many cell free translation systems (Revel and Groner, 1978). It has recently been shown that reovirus transcription during the first four hours in infected cells produces capped and methylated mRNA's, and that these are translated more efficiently in extracts of uninfected cells than in infected cells. The later stage of mRNA production provides only uncapped mRNA's, as the immature particles which transcribe them do not express their guanylyltransferase and methylase activities (Skup and Millward, 1980), and these uncapped mRNA's are translated more efficiently in infected cells than in uninfected cells. The uncapped mRNA molecules

1.3.4.3.2 (Cont'd.)

presumably have a different mechanism of initiation from early mRNA, as the efficiency of translation in this second stage of synthesis appears to be high. This may improve competition between viral and cellular messengers on ribosomes which have already met capped viral messengers. This is a novel control mechanism and is not yet understood.

A common modification of eucaryotic mRNA is a 3' poly A tail, and this is thought to affect translation of mRNA's by improving their stability and their ability to sustain long term translation. Reovirus mRNA lacks 3' poly A, but has been shown to be extremely stable when injected into *Xenopus* oocytes (McCrae and Woodland, 1981) so must have other means of maintaining its general stability. However, the possibility that poly A prevents 3-5' exonuclease binding and so protects the RNA against nuclease digestion has not been ruled out.

1.3.4.3 Reovirus translation in vitro

Cell free translation of reovirus mRNA was first achieved in cytoplasmic fractions of reovirus infected L cells containing virus-specific polysomes (McDowell and Joklik, 1971), and the synthesis of 6

1.3.4.3. (Cont'd.)

structural polypeptides and two non-structural polypeptides was identified. In later studies, exogenous reovirus mRNA was translated in mammalian cell-free extracts (Levin et al., 1971; Graziadei and Lengyel, 1972; McDowell et al., 1972). McDowell et al. (1972) found that larger mRNA's were translated most effectively in rabbit reticulocyte lysates and they identified 8 primary gene products. Using higher resolution gel analysis, Both et al. (1975) found all 10 primary gene products after translation in a wheatgerm system. The primary gene products were confirmed by their comigration with viral proteins and by their molecular weight which was consistent with the coding potential of the mRNA's.

More detailed studies on initial steps of translation have been carried out by Kozak and Shatkin (1977b, 1978), and these have general relevance in eucaryotic translation systems. Kozak and Shatkin's observations followed the earlier ones of Levin et al. (1972), who showed that all three size classes of mRNA formed complexes with ³⁵S-methionyl tRNA and salt washed 40S and 60S ribosomes at a puromycin sensitive ribosome site. Kozak and Shatkin (1977b) subsequently demonstrated that the first step in translation was the binding of the 40S ribosomal subunit at the 5'

1.3.4.3. (Cont'd.)

terminus of mRNA. The ribosome never bound to internal regions, and had greater affinity for capped than uncapped mRNA, although it also bound significant quantities of uncapped mRNA. The 40S subunit covered about 50-60 residues along the 5' end. Once bound, the 40S subunit moved along the mRNA to the first initiation codon, and then combined with the 60S subunit, methionyl tRNA and initiation factors. The secondary structure of the mRNA was important for the binding of the 60S subunit. Translation then proceeded normally: the later stages of in vitro translation have not been studied in detail.

1.3.4.4. Gene-protein coding assignments of reovirus

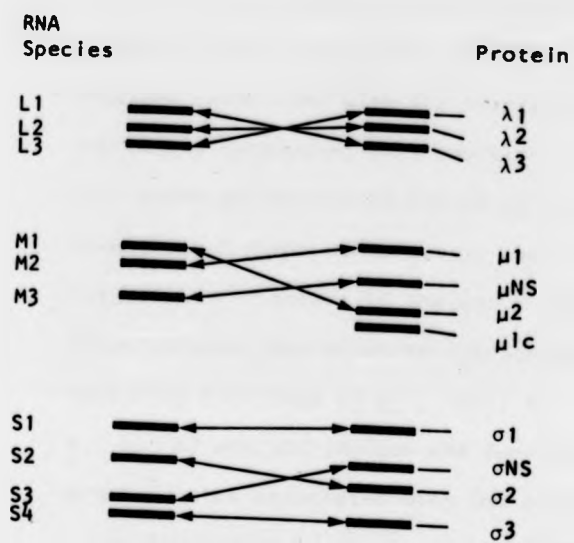
As reovirus RNA is segmented, it was theoretically possible to translate each individual mRNA species in vitro and determine its protein product. The main difficulty was the isolation of homogeneous preparations of each mRNA, particularly the larger species in sufficient quantity. In a new approach, McCrae and Joklik (1978) isolated the 10 individual genome segments, denatured the dsRNA in 90% dimethyl sulphoxide at 50°C and then diluted them into a wheatgerm translation system, before renaturation could occur. This approach was successful - the dsRNA molecules were translated into complete proteins, and the polypeptide encoded by each

1.3.4.4. (Cont'd)

genome segment was therefore determined. The gene-coding assignments found by this group are summarised in Fig.11. Mustoe et al. (1978) obtained the same results in reassortment experiments. The genes and proteins of the three serotypes of mammalian reovirus have detectable size differences, and in most reassortants the origin of each gene (and its protein) can be determined. Mustoe et al. (1978) produced reassortants between the three serotypes and correlated the changes in migration of genome segment with the corresponding changes in protein migration.

Recently, Levin and Samuel (1980) have determined coding assignments of the four small RNA species by the direct method of isolating individual mRNA molecules and translating each one. Their results agree with those of McCrae and Joklik (1978) and they were also able to determine the relative translational efficiencies of each mRNA. The translational efficiency of each mRNA agreed with results obtained in vivo, suggesting that competition between the s-mRNA species is insignificant and that the sequences determine translational efficiency.

Fig.11 Gene-coding assignments of reovirus proteins
(from McCrae and Joklik, 1978)



1.3.4.5 Reovirus morphogenesis

1.3.4.5.1 Formation of progeny dsRNA

Schonberg et al. (1971) demonstrated that the reovirus plus stranded transcripts (mRNA) served as templates for transcription of minus strands, which remained associated with the template forming dsRNA. The enzyme catalysing this reaction is labelled the ss \rightarrow dsRNA polymerase (Zweerink et al., 1972). Formation of dsRNA commences at about 4h after infection. It occurs in the early immature particles which contain plus stranded transcripts in RNAase sensitive form (Acs et al., 1971) surrounded by λ_1 , λ_2 , σ_2 and μ_1c (Morgan and Zweerink, 1974). The dsRNA remains associated with the particles and is never found free in the cytoplasm (Gomatos, 1967). Equal numbers of all species of dsRNA are formed (Watanabe et al., 1968): the mechanism by which each particle receives one of each of 10 RNA molecules is as yet unknown. Synthesis of s, m and ℓ dsRNA's in particles is sequential (Zweerink, 1974).

1.3.4.5.2 Assembly of particles

The most immature virion precursors are the particles within which dsRNA is formed, consisting of RNAase sensitive complexes of 10 single stranded RNA species and some virus specified polypeptides.

1.3.4.5.2 (Cont'd.)

These particles are unstable and heterogeneous in nature so it has been difficult to purify them. The particles increase in sedimentation coefficient sequentially as each size class of dsRNA is made. Molecules of the S, M and L classes are formed in particles that sediment at about 250, 450 and 550S respectively (Zweerink, 1974). The most stable of these subviral particles resemble cores, contain dsRNA and the same proteins as in early SVP's, namely λ_1 , λ_2 , σ_2 and μ lc (Morgan and Zweerink, 1974) and are responsible for late transcription. This series of core-like particles accumulate in cells infected at 39°C, and are the products of infection with certain ts mutants (Fields et al., 1971; Morgan and Zweerink, 1974).

The mechanism of maturation of these core-like particles into complete virions is unknown. The final stage appears to be addition of the polypeptide σ_3 , which abolishes transcriptase activity (Astell et al., 1972). Virus isolated from infected cells which were pulse-labelled very briefly just prior to disruption are labelled only in σ_3 (Joklik, 1981). Parental SVP's also acquire σ_3 (and σ_1), and these are liberated with the newly formed progeny particles at the end of the replication process. These parental

1.3.4.5.2 (Cont'd.)

particles still lack part of plc and their infectivity has not been determined.

1.3.4.6 Reovirus pathogenesis

Reoviruses (Respiratory Enteric Orphan Viruses) as the acronym reminds us are not associated with any particular disease in man and little work has been done on their pathogenesis. They are occasionally associated with respiratory disease, but are essentially enteric. Some detailed pathological studies have recently been made because of the current interest in the molecular basis of reovirus virulence. Despite the fact that serotype 3 has been associated with hepatitis and diarrhoea in mice, only serotype 1 has been seen to multiply in intestinal tissue. The primary site of multiplication is unknown, but the viruses enter M cells of the intestinal epithelium and spread from there into Peyer's patches. M cells overlie Peyer's patches in the intestine. After replication in the intestine, the virus spreads to more distal sites, and eventually infects the brain tissue (serotypes 1 and 3). Serotype 1 is associated with non-lethal hydrocephalus and infects ependymal cells. Serotype 3 causes acute, lethal encephalitis and infects only neuronal cells, and never ependymal cells. The molecular basis for the different tissue tropism

1.3.4.6 (Cont'd.)

of the virus is controlled by the σ 1 protein, as described in section 1.4.1.

1.3.5 Rotavirus Replication

Rotaviruses generally grow poorly in cultured cells, but as they multiply well in the small intestine of their animal host much of the ultrastructural study of rotavirus replication and morphogenesis in infected cells has been done in the animal host. The current knowledge of their multiplication in cell culture and animal hosts is described below.

1.3.5.1 Factors affecting rotavirus growth in tissue culture

The difficulty in growing rotaviruses in tissue culture has been one of the greatest stumbling blocks in rotavirus research. SA-11, several calf rotaviruses, porcine rotaviruses and avian rotaviruses have now been adapted to growth in cell cultures (Malherbe and Strickland-Cholmley, 1967; Mebus et al., 1971; Welch and Twiehaus, 1973; Bridger and Woode, 1975; L'Hardion and Scherrer, 1976; Chasey, 1977; Thiel et al., 1977; McNulty et al., 1979). The growth of human rotavirus in primary cultures of African green monkey kidney cells was achieved only after 11 passages in gnotobiotic piglets (Wyatt et al., 1980).

1.3.5.1 (Cont'd).

Rotaviruses infect villi of the small intestine in vivo, so the reason for their inability to grow in tissue culture was originally thought to be due to the fact that they only infected differentiated cells. However, growth in continuous cell lines has now been achieved. As viruses were often observed on the microvillous brush border, it was suggested that the lactase present there may act as receptor and uncoating enzyme for rotaviruses (Holmes et al., 1976). However, non-adapted viruses often infect cells without lactase on their surface and synthesise virus specific protein (McNulty, 1978; Thouless, 1979), so the block to growth appears to be later in infection. McNulty (1978) has observed massive production of coreless, defective particles on non-productive infection. Pancreatic enzymes were also postulated to be important for infectivity (Thiel et al., 1977, 1978). These will be discussed in the next section.

1.3.5.2 Adsorption, uptake and uncoating in infected cells

Rotavirus adsorption onto permissive cells occurs at 4°C or 37°C and is routinely allowed one hour to complete. An interesting feature of rotavirus adsorption to cells is that it appears to be greatly enhanced by low-speed centrifugation during the adsorption period

1.3.5.2 (Cont'd).

(Banatvala et al., 1975; Bryden et al., 1977), especially with rotaviruses not adapted to tissue culture. This process possibly concentrates viral aggregates onto the cells and filtration of the virus stock prior to adsorption reverses the effect (Schoub et al., 1979). No observations on the uptake of rotaviruses into cells have been made, but partial uncoating within infected cells has been demonstrated (Clark et al., 1980). This 'uncoating' involves removal of three outer shell proteins, an increase in density of 0.02 g/ml and the transcriptase is activated. The process is therefore analogous to the mechanism of transcriptase activation in vitro (Cohen, 1977). The inner shell of the virus is not disrupted during uncoating.

Virus infection of cells in tissue culture was shown to be greatly enhanced by the presence of pancreatin or trypsin in the culture medium (Almeida et al., 1978; Theil et al., 1978). Pretreatment of cells with trypsin did not enhance infectivity, but pretreatment of virus did (Babiuk et al., 1977; Almeida et al., 1978; Barnett et al., 1979; Clark et al., 1979; Schoub et al., 1979). Pretreatment of purified virus preparations also enhanced their infectivity, indicating that it did not simply digest viral inhibitors in the growth medium

1.3.5.2 (Cont'd.)

(Graham and Estes, 1980). Clark et al. (1981) found that trypsin treatment of virus had no effect on dispersing viral aggregates or on the attachment of virus to cells, but that it enhanced infectivity by allowing a greater fraction of parental virus to uncoat after infection. 44% of trypsin-treated particles had uncoated by 1h after adsorption, compared with 15% of untreated particles, and trypsin-treated particles also synthesised 2-fold more viral RNA than untreated particles. Trypsin treatment of virus particles cleaves one of the outer shell polypeptides (VP3) into two smaller proteins, but does not itself cause uncoating. Other proteolytic enzymes with the same effect include pancreatin and elastase, but chymotrypsin and lactase do not enhance infectivity (Estes et al., 1981). Some of VP2 is also cleaved by some of the enzymes, such as trypsin, but these cleavages do not affect infectivity.

1.3.5.3 Adsorption, uptake and uncoating in the animal host

Rotaviruses specifically infect the small intestinal villus epithelium of young mammals (McNulty, 1979). Most are found in the tall columnar cells, rather than cuboidal or squamous epithelial cells, and have also been identified in sub-epithelial macrophages, reticular cells of the villus lamina propria and upper mesenteric lymph node (Stair et al., 1973; Mebus et al., 1977). The nature of the receptors or the mechanism of entry is not known but viruses have been observed on the microvillus brush border and are therefore probably phagocytosed.

1.3.5.4.1 Synthesis of rotavirus proteins in vivo

The first reports of rotavirus protein synthesis in infected cells came from Thouless (1979) and Matsuno and Mukoyama (1979). They compared structural polypeptides with those produced in infected cells pulse labelled with ^{35}S -methionine 2-10h post infection. Virus protein synthesis was directly detectable by 4h after infection and predominated over cellular protein synthesis by 6h after infection. Thouless identified production of all the structural polypeptides reported previously (Table 5, p⁵⁰) except VP4.2 and VP7.2 and observed production of each to start at about the same time. Two polypeptides migrated closely in the region of VP3 (namely VP3 and VP4). In addition, Thouless identified three non-structural polypeptides - two migrated just faster than VP7, had molecular weights of 33K and 31K and were called VP8 and VP9, and one migrated ahead of VP10 and had a molecular weight of 17K. Matsuno and Mukoyama did not resolve VP3 and VP4 or recognise VP8 and VP9, possibly due to poor gel resolution, but found an additional non-structural polypeptide of 13.5K. Both groups identified VP5 as non-structural, but Thouless suggested it was a precursor of VP4.2. These findings are summarised in Table 5^(p50). Urquidí et al. (1981) described the same basic polypeptide pattern with only two non-structural polypeptides, VP5 and VP8. The gel resolution was insufficient to differentiate VP7, VP7.1, VP8 and VP9 or the two infected cell proteins VP3 and VP4.

1.3.5.4.1 (Cont'd.)

McGrae and Faulkner-Valle (1981) resolved all the infected cell polypeptides described previously and noted several posttranslational modifications, discussed in section 1.3.5.4.2. Arias et al. (1982) confirmed previous observations on these polypeptides.

No studies have been undertaken on the relative efficiencies of translation of each mRNA species, but ³⁵S-methionine appears to incorporate most strongly into VP6, followed by VP7, VP8, VP10, VP2 and VP3 in approximately that order and comparison of ³⁵S methionine-labelled and stained structural proteins show an approximate correlation (described in chapter 4 of this Thesis). VP1, VP9 and VP11 were poorly translated, and VP5 forms a diffuse band. Figure 1 of chapter 4 illustrates the relative amounts of protein in infected cells. As rotavirus mRNA seems to be transcribed in vitro in a similar manner to reovirus mRNA with fewer high than low molecular weight transcripts being produced, these differences in protein quantity probably indicate that some translational controls are operating.

1.3.5.4.2 Studies on modifications of rotavirus polypeptides

Recent studies on rotavirus polypeptide synthesis in vivo have improved the understanding of rotavirus polypeptides by defining several post-translational modification steps involved in the production of infectious virus.

- a) Trypsin-induced cleavages. Matsuno and Mukoyama (1979) examined the effect of trypsin on the polypeptide composition of purified rotavirus and, while they identified several new polypeptide bands, especially of lower M.W., and observed decreased quantities of VP3 and VP4.2, they could not associate the trypsin-induced enhancement of infectivity with proteolytic cleavage of any ^{one} polypeptide. Later, Espejo et al. (1981) using SA-11 clearly showed that trypsin caused the specific cleavage of polypeptide VP3 into two smaller polypeptides they called VP5 (VP4.2 in our nomenclature) and VP8 (VP4.3), and that this proteolytic cleavage was associated with the enhancement of infectivity. This was confirmed by single dimension peptide mapping and also in later reports by Estes et al. (1981); Clark et al. (1981) and Dyal-Smith and Holmes (1981).

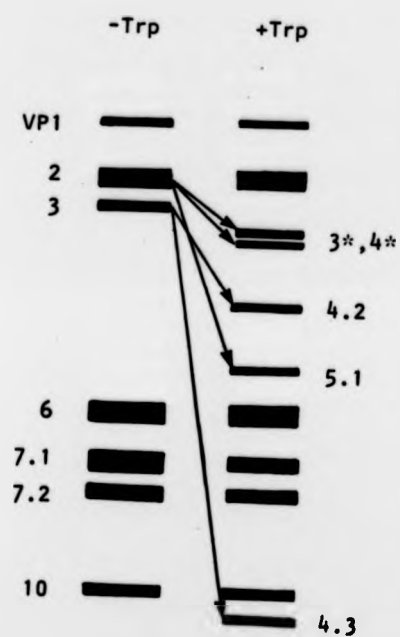
1.3.5.4.2 a) Trypsin-induced cleavages (Cont'd).

Estes et al. (1981) also identified VP3*, 4* and a 53K molecular weight polypeptide (previously called VP3, VP4 and VP5 [McCrae and Faulkner-Valle, 1981]) as trypsin cleavage products of VP2, although not all VP2 became cleaved. This means that VP3* is a different polypeptide from VP3 and this fact may clarify the question of whether rotavirus had two structural polypeptides in the VP3 region or a single one. The 53K cleavage product comigrates with a primary gene product (VP5) also seen in infected cells, but Estes et al. (1981) showed by single dimension peptide mapping that the 53K cleavage product and the 53K primary gene product (VP5) are unrelated, and that VP3*, VP4* and 53K shared many common peptides with VP2. The proteases pancreatin and elastase were also shown to enhance infectivity - these cleaved VP3 to produce VP4.2 but formed other small cleavage products and not VP4.3. Estes et al. (1981) therefore concluded that VP4.2 was the cleavage product of biological importance. Chymotrypsin was also shown to cleave VP3 but did not enhance infectivity: VP3 was cleaved into VP4.2 and VP4.3, but VP4.2 was then further degraded into smaller fragments. This could explain the observed

1.3.5.4.2 a) Trypsin-induced cleavages (Cont'd).

loss of VP4.2 by Matsuno and Mukoyama (1981) in trypsin treated virus if their trypsin preparation was contaminated with chymotrypsin. Their virus inoculum presumably contained some trypsin-like protease since VP4.2 was present. Clark et al. (1981) studied the effect of trypsin on purified calf rotavirus and showed that VP3 was cleaved into polypeptides of molecular weight 67K and 20K. The smaller cleavage product was smaller than that reported for SA-11 by Espejo et al. (1981) and Estes et al. (1981) but they also claimed that the M.W.'s of the other smaller proteins were consistently lower than those reported elsewhere (Thouless, 1979; McCrae and Faulkner-Valle, 1981; Novo and Esparza, 1981). The three independent studies described in this section present evidence for biologically important trypsin-induced cleavage of VP3 into defined products as well as cleavage of VP2. The trypsin-induced cleavages are summarised in fig.12.

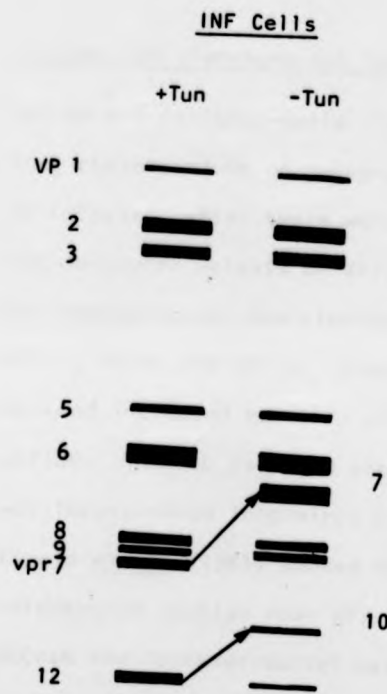
Fig.12 Trypsin-induced cleavages of rotavirus proteins



1.3.5.4.2 b) Glycosylation. Rodger et al. (1977) initially identified the structural polypeptide VP7.1 as a glycoprotein by periodic acid-Schiff staining. Later, Matsuno and Mukoyama (1979) with ^{14}C -glucosamine labelling showed that their VP7 (our VP7.1: see Fig. 11) was glycosylated. A more convincing study by McCrae and Faulkner-Valle (1981) was undertaken by pulse-labelling polypeptides in infected cells with ^3H -glucosamine and also by pulse labelling the polypeptides in the presence of the glycosylation inhibitor, triniticamycin. They identified VP7 (own nomenclature) as a glycoprotein and its precursor as vpr7 (Fig. 12) and found a second glycoprotein VP10 derived from VP12. Arias et al. (1982) confirmed these results and also showed that in both cases the oligosaccharides were cleavable by endoglycosidase H. (endo- β -N-acetylglucosaminidase H), an enzyme that hydrolyses mannose-rich chains from glycoproteins (Tarentino and Maley, 1974), leaving the unglycosylated precursor. This indicated that the oligosaccharides on these glycoproteins were rich in mannose. Estes et al. (1981) purified virus grown in the presence of ^3H -glucosamine and identified VP7 and VP7a (our VP7.1 and VP7.2) as glycoproteins. They did not identify VP10 in purified virus particles.

Fig.13

Glycosylation of rotavirus proteins



1.3.5.4.2 b) Glycosylation (Cont'd.)

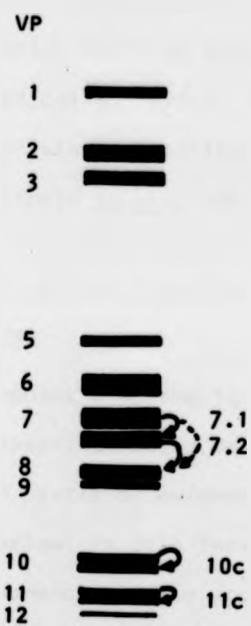
unlike McCrae and Faulkner-Valle (1981), possibly because it is a very minor component and weakly labelled. The glycosylation steps are summarised in figure 13.

c) Polypeptide cleavages not induced by trypsin.

McCrae and Faulkner-Valle (1981) showed that during long chase periods of polypeptides pulse-labelled in infected cells, there were minor reductions in the molecular weights of VP7, VP10 and VP11. In our nomenclature, the cleavage products are called VP7.1, VP10c and VP11c. Urquidi et al. (1981) also noticed increased mobility of VP10 after a chase period. In each case the smaller cleavage product was incorporated into virus particles. Additionally, Espejo et al. (1981) showed that the structural polypeptide smaller than VP7.1 (labelled VP8 by McCrae and Faulkner-Valle) was closely related to VP7 as determined by single dimension peptide mapping. This suggests that VP8 seen in infected cells is non-structural and the structural polypeptide that almost comigrates with VP8 is a further post-translational modification of VP7.1 or of VP7. We call this polypeptide VP7.2 (see fig.14). Dyal-Smith and Holmes (1981) confirmed, by single dimension peptide mapping

Fig.14

Natural cleavages of rotavirus proteins



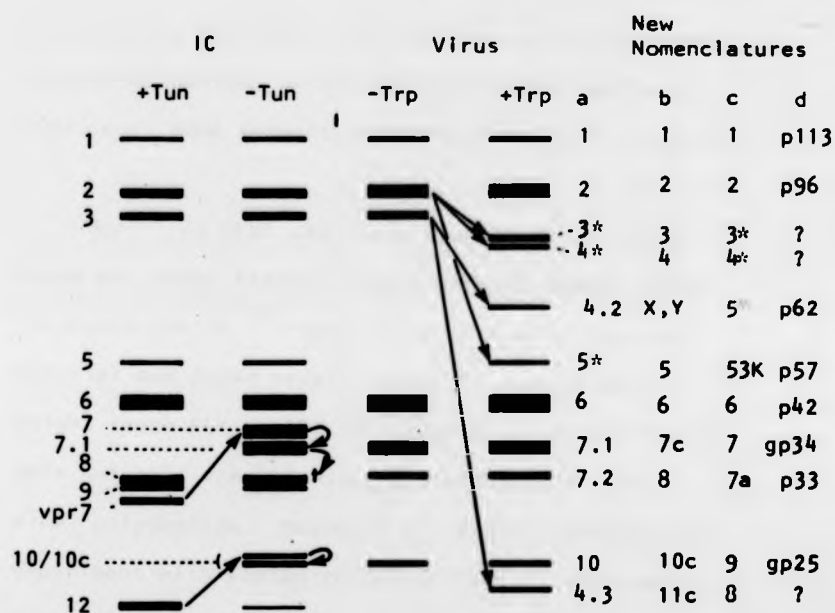
1.3.5.4.2 c) (Cont'd.)

of the viral proteins, the relationship between polypeptides VP10 and VP10c and between VP7 and VP7.1 as well as VP8 and VP9. There was also some similarity between profiles of VP7.1 and VP8, although this was less significant than that of VP7/VP7.1 and VP8/VP9. A possible explanation is that their VP8 consists of a mixture of VP8 and of VP7.2 which would be expected to comigrate in infected cells. VP7.1, VP7.2, VP10c and VP11c are found in virus particles (McCrae and Faulkner-Valle, 1981; Espejo et al., 1981).

1.3.5.4.3 Conclusions on modifications of rotavirus structural polypeptides.

Fig.15 proposes a scheme for the modification of rotavirus polypeptides based on the literature reviewed above, and partly on evidence presented in the Results Section (below) in this Thesis. It also proposes a modified nomenclature to account for recent published results, and compares it with the nomenclature of Estes et al., (1981), McCrae and Faulkner-Valle (1981) and Dyal-Smith and Holmes (1981). Another interesting feature of rotavirus proteins, recently reported by Estes et al. (1982), is the variation in size of VP7 in different isolates of SA-11. This is fully discussed in chapter 4. We also suspect variation in VP4.2 or a complicated series of steps involved in its formation as discussed in chapter 5.

Fig.15 Summary of the modification steps involved in the maturation of rotavirus proteins



Current status of the nomenclature and processing of rotavirus polypeptides (VP) in infected cells and virus particles. Arrows indicate putative processing steps through proteolysis or glycosylation which have been demonstrated by treatment of virus with trypsin (Trp) or of infected cells with tunicamycin (Tun) respectively. Note that VP7.2 in infected cells (+) is only seen after immune precipitation with α VP7.1 or α VP7.2 as it is obscured by the more abundant VP8. The nomenclature used in this report is indicated under heading (a), that of McCrae and Faulkner-Valle (1981) in (b), that of Estes *et al.* (1981) in (c) and that of Dyall-Smith and Holmes (1981b) in (d). In our nomenclature we use * to indicate cleavage products of VP2.

1.3.5.4.4. In vitro translation of rotavirus mRNA

In vitro translation studies with rotavirus mRNA has focussed on translation of mRNA transcribed in vitro under reaction conditions similar to those optimised for reovirus. In all cases, studies on in vitro translation were carried out to identify the primary gene products, i.e. no work on translational control mechanisms or translational efficiencies of individual mRNA segments has been done with rotavirus.

Rotavirus mRNA was first translated in vitro by Cohen and Dobos (1979). These workers demonstrated incorporation of ^{35}S -methionine into acid-insoluble material and found several bands in the molecular weight range 44K to 12K by analysis on polyacrylamide gels but only the 44K M.W. band comigrated with a viral polypeptide. Mason et al. (1980) repeated the experiment with simian rotavirus (SA-11) mRNA and identified eleven primary gene products of molecular weight range 125K to 12K, many of which comigrated with the viral proteins. Four of these polypeptides comigrated with viral structural proteins - VP1, VP2, VP3 and VP6 - and a further four comigrated with polypeptides seen in infected cells - VP5, VP8, VP9 and VP12. The other three polypeptides did not comigrate with viral proteins - one of these was just smaller than VP9, and the other two were smaller than

1.3.5.4.4. (Cont'd).

VP10. Several polypeptides found in vivo were not found in vitro, including VP7/VP7.1 and VP10/VP10c. This implies that at least two of the primary gene products are post-translationally modified into viral polypeptides in infected cells. These and other post-translational modifications are described in section 1.3.5.4.2.

Analysis of the products of in vitro transcription had showed that the amount of each mRNA produced decreased as its size increased (Bernstein and Hruska, 1981b) the possibility being that each mRNA was transcribed at the same rate as in the case of reovirus. Evidently, translation of each polypeptide did not occur at the same rate, as some polypeptides were produced in greater abundance than others, as discussed in Chapter 4 of this Thesis. There has been no published analysis on the relative translation rates of each mRNA species.

The question of which polypeptide each mRNA codes for has been the subject of intense research. Mason et al. (1980) fractionated mRNA on sucrose gradients, and translated individual fractions in vitro. This showed that fractions containing smaller mRNA's produced the smaller proteins, while fractions containing larger mRNA's produced the larger proteins.

1.3.5.4.4. (Cont'd).

There have also been several reports on gene coding assignments of each of the rotavirus genome segments, as identified by in vitro translation (Smith et al., 1980; Dyall-Smith and Holmes, 1981b; Arias et al., 1982; McCrae and McCorquodale, 1982). In all cases, denatured dsRNA was translated in wheatgerm or rabbit reticulocyte lysates as described by McCrae and Joklik, (1978). For simplicity, all descriptions below will use the protein nomenclature of McCrae and Faulkner-Valle (1981). Smith et al. (1980) translated isolated species of the six largest genome segments of SA-11 in a wheat germ translation system, and found a direct size correlation between the polypeptides and the gene segments they were translated from, i.e. VP1 was assigned to segment 1, VP2 to segment 2, VP3 to segment 3, VP4 to segment 4, VP5 to segment 5 and VP6 to segment 6. However, VP3 and VP4 have now been reversed - the VP4 of McCrae and Faulkner-Valle (1981) became the viral protein VP3 of Espejo et al. (1981) and Estes et al. (1981) mainly because the 'VP3' of McCrae and Faulkner-Valle (1981) was not seen in virus particles and was not resolved from 'VP4' in infected cells. Thus the gene product of segment 4 is now called VP3. Dyall-Smith and Holmes (1981) extended the study to include genome segments 10 and 11 which coded for VP11 and VP12 respectively, although

1.3.5.4.4 (Cont'd.)

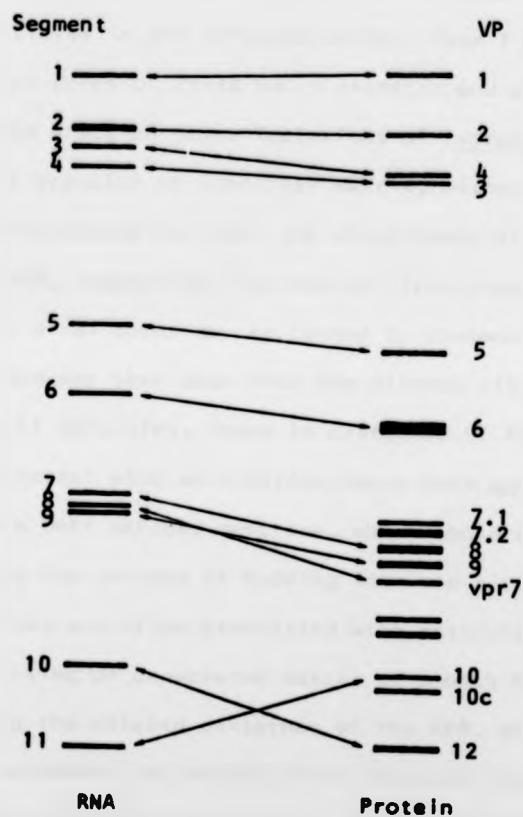
In some human strains (which are said to have a 'short' electropherotype as the RNA segments cover a shorter size range) the genome segments 10 and 11 are reversed in mobility. McCrae and McCorquodale (1982) using rabbit reticulocyte lysate confirmed these results for calf rotavirus, showing that the electropherotype of calf rotavirus is the same as that of human type 1 (the 'short' electropherotype) and they also determined the proteins coded by genome segments 7, 8 and 9. Segment 7 coded for VP8, segment 8 for vpr7 (the precursor of VP7) and segment 9 for VP9. Segments 7 and 8 appeared to produce a mixture of the three polypeptides but were assigned on the basis of the major product. Segment 3 produced a protein that did not comigrate with any of the virus proteins and it was postulated to code for a precursor of VP3. Arias et al. (1982) produced similar, but less clear, results for VP1, 2, 3, 4, 5, 6, 9, 10 and 11 but could not distinguish the assignments of RNA segments 7 and 8. Interestingly, the latter two groups both assigned gene segment 9 to the non-structural polypeptide VP9, although Kalica et al. (1981) assigned gene segment 9 to code for the neutralization-specific antigen of human rotavirus by recombination studies. It is possible that gene segments 8 and 9 are reversed

1.3.5.4.4 (Cont'd).

in order between human and calf rotaviruses. A description of gene coding assignments is shown in Fig. 16. The protein nomenclature of Smith et al. (1980), Arias et al. (1982) and our own are in columns beside that of McCrae and Faulkner-Valle (1981).

Fig.16

Schematic diagram to show the complete RNA-protein-coding assignments for the U.K. tissue culture adapted calf rotavirus. From McCrae and McCorquodale (1982).



1.3.5.5. Rotavirus morphogenesis

1.3.5.5. a) Rotavirus morphogenesis in the animal host

Within infected villus epithelial cells, virus particles are usually found within dilated cisternae of rough endoplasmic reticulum (RER) (McNulty, 1979). They have also been noted in cytoplasmic vesicles within sub-epithelial cells and in large vacuoles. Chasey (1977) observed 5 different types of rotavirus particles in the infected cells. Type I particles resembled virus cores of 25-30 nm in diameter and were associated with electron dense inclusions of 'viroplasm'. Viroplasm is a granular or fibrillar matrix, without a membrane and is outside but near the virus-containing cisternae of RER, suggesting its role as virus precursor material. The viral cores may be formed by condensation of viroplasm and may then pass into the dilated cisternae of RER. Type II particles, found in cisternae of RER, were 70-80 nm in diameter with an electron dense core and were surrounded by a well defined membrane, which could be acquired during the process of budding into the cisternae. Type II particles are often associated with reticular inclusions consisting of convoluted masses of smooth membrane found bordering the dilated cisternae of the RER, and these are either a by-product of budding or a source of the membrane surrounding enveloped virus. Saif et al. (1978) described budding of enveloped virus particles at the periphery of this inclusion material. Type III particles were smaller than type II (50-65 nm) and had an indistinct outline, and no envelope. Type IV particles have only been

1.3.5.5 a) Rotavirus morphogenesis in the animal host (Cont'd).

described by Chasey (1977) and are only found in cells that did not appear to contain types I, II and III particles and were seen in large vacuoles, suggesting perhaps that they were formed under adverse conditions. Type V particles were 50 nm in diameter and consisted of an electron dense core surrounded by an electron-lucent region and then a denser outside. These particles were interconnected by thin filaments, possibly nucleic acid. Type V particles have been seen in vacuoles, cisternae of RER and in the intestinal lumen. The relationship of any of these types of particle to intact and single shelled particles seen by negative staining is not known. Two other types of inclusion material have also been found. Electron dense inclusions of circular or paired ring structures free in the cytoplasm and linear arrays of tubular like components, along which core like particles of 33 nm were found, had been noted in villous epithelial cells. Single and double membrane tubules have also been described in the cytoplasm and cisternae, and single membraned tubules have been seen in nuclei. Single and double membraned tubules have diameters within the size range for non enveloped and enveloped virus particles respectively, and may later segment into virus particles or represent defective formation of virus particles corresponding to the tubules of capsid protein seen by negative staining.

1.3.5.5 a) Rotavirus morphogenesis in the animal host (Cont(d.)

There is no information on molecular steps involved in the morphogenesis of rotavirus.

1.3.5.5 b) Rotavirus morphogenesis in cell cultures

Calf, human, pig, monkey and turkey rotavirus multiplication has been studied by electron microscopy in cell cultures (McNulty et al., 1976b; Wyatt et al., 1976; Saif et al., 1978; Lecatsas, 1972; McNulty et al., 1979). The viruses were found predominantly in the cisternae of the RER with dense inclusions of viroplasm outside the cisternae. Enveloped and non-enveloped types of particle were seen in these cells and intranuclear tubules were also found. Cell cultures infected with turkey rotavirus contained type 1 particles but little viroplasm, as well as a lot of type II particles and the membrane inclusions, from which type II particles appeared to be budding. Double membraned tubules were found in cisternae of RER and single membraned tubules free in cytoplasm. Nuclear tubules were seen but were smaller than those seen with mammalian rotaviruses, being only about 25 nm in diameter. Virus particles were released through breaks in the plasma membrane of disintegrating cells.

A recent study by Petrie et al. (1982) used immunoperoxidase staining with monospecific antisera to identify the subcellular locations of different antigens during

1.3.5.5 b) (Cont'd.)

morphogenesis. Antiserum against complete virus stained the cytoplasm, especially viroplasmic inclusions and the membranes of cisternae of RER. In contrast, α VP2 and α VP6 stained the cytoplasm and viroplasmic inclusions while α VP7.1/7.2 did not stain cytoplasm or viroplasm but did stain the RER and also the nuclear membrane very strongly. They concluded that VP2 and VP6 were synthesised in the cytoplasm and concentrated into immature virus particles in viroplasmic inclusions, while VP7 was glycosylated in the lumen of the RER and the single shelled particles acquired VP7 (and presumably the outer shell) as they budded into the RER.

Rotavirus morphogenesis appear to be unique among the reoviruses and orbiviruses. All these viruses develop within intracytoplasmic matrices of viroplasm and are associated with swelling of the ER. However, only rotaviruses are found in large numbers in distended cisternae of RER, and only rotaviruses and a few orbiviruses (Murphy *et al.*, 1968; Bowne and Ritchie, 1970) form enveloped particles by budding. The orbiviruses differ also by budding from the plasma membrane. Cytoplasmic tubules are also seen in orbivirus infections but nuclear filaments are only seen in Colorado tick fever infection (Murphy *et al.*, 1968). Reoviruses are unique in that the developing particles are associated with the mitotic (and non-mitotic) spindle microtubules (Anderson and Doane, 1966; Sharpe and Fields, 1981).

1.4.1 Reovirus Genetics

The use of temperature sensitive (ts) mutants and reassortment of gene segments between serotypes have provided much information on the functions of specific reovirus genes and the same approach is now being applied to the study of rotavirus gene function (Kalica et al., 1981).

1.4.1.1 Production and characterisation of ts mutants of reovirus

Ts mutants were isolated by Fields and Joklik (1969) and Ikegami and Gomatos (1968). The mutants of Fields and Joklik (1969) were divided into seven groups by reassortment experiments. A further three groups have now been isolated (Ramig and Fields, 1979). Each of these groups corresponds to a ts lesion on a different gene segment, so all 10 reovirus gene segments are represented. Table 9 summarises results from several publications. Extragenic suppression of ts lesions is very common in reovirus - i.e. a second "suppressor" mutation arises elsewhere in the genome which reverts the effect of the ts lesion. 25/28 revertants analysed by Ramig and Fields (1979) were extragenically suppressed, and the later groups of ts mutants (Ramig and Fields, 1979) were isolated from mutants under extragenic suppression. This phenomenon is important because these suppressor mutations probably act by altering another protein which interacts physically with the protein

TABLE 9 **Reovirus Temperature sensitive mutants ^a**

Reassortment Group	Type	Lesion in gene
A	Late	L2 or M2
B	Late	L2
C	Early	S2
D	Early	M2 or L1
E	Very Early	S3
F	Late	?
G	Late	S4
H	?	?
I	?	?
J	?	?

a) Mutant groups were isolated as follows:

A to E by Fields and Joklik (1969)

F and G by Cross and Fields (1972)

H to J by Ramig and Fields (1979)

Very early: before transcription of mRNA

Early: before formation of progeny dsRNA

Late : after formation of progeny dsRNA

1.4.1.1 (Cont'd.)

encoded by the mutant gene. Characterisation of the pairs of mutations in 'pseudorevertants' could provide information on interactions between the reovirus proteins, and this information would be useful in morphogenetic studies.

1.4.1.2 Identification of the functions of individual reovirus-coded polypeptides

The functions of many reovirus proteins have been determined by genetic analysis of reassortant viruses from two different serotypes. The method was useful and highly successful for various reasons:

- a) Genetic reassortment occurs at high frequency.
- b) The RNA's of the three serotypes all vary in electrophoretic mobility (Sharpe et al., 1978), and this allows identification of the origin of each RNA segment in reassortants.
- c) The three serotypes vary in haemagglutination specificity and pathogenic properties (Eggers et al., 1962; Raine and Fields, 1973), and are distinguishable by neutralization.
- d) Rescue of the ts phenotype is a useful selection method for reassortant viruses.

1.4.1.2 (Cont'd.)

- e) The polypeptide encoded by each RNA segment has been identified (Mustoe et al., 1978; McCrae and Joklik, 1978).

1.4.1.2.1 Identification of the viral haemagglutinin

Reovirus haemagglutinin is type specific: type 1 agglutinates human but not bovine erythrocytes and type 3 agglutinates bovine erythrocytes but rarely human erythrocytes (Eggers et al., 1962). Reassortants were derived from type 3 and type 1 reoviruses and their RNA profiles and haemagglutination properties were analyzed. Each of the six reassortants analyzed behaved either as type 1 or type 3 in haemagglutination assays and none agglutinated both types of erythrocytes. In all cases the type of agglutination was the same as that of the parent that had contributed the S1 gene segment, which codes for the outer capsid polypeptide $\sigma 1$ (Weiner et al., 1978). This polypeptide comprises only 2% of the outer capsid of reovirus, while $\mu 1c$ and $\sigma 3$ comprise 38% and 60% respectively.

1.4.1.2.2 Identification of the reovirus neutralization-specific antigen

Genetic analysis of reassortant viruses also showed that type-specific viral neutralization was mediated

1.4.1.2.2 (Cont'd).

through $\sigma 1$, which is encoded by RNA segment S1 (Weiner and Fields, 1977). Therefore, this minor polypeptide must be exposed to antibody on the reovirus outer shell, a prediction which has been confirmed by ^{125}I labelling. Recent studies with monoclonal antibodies (Hayes *et al.*, 1981) have also implicated $\sigma 3$ and $\lambda 2$ as neutralization-specific antigens although neutralization with $\alpha\sigma 3$ and $\alpha\lambda 2$ was not type-specific.

1.4.1.2.3 Identification of polypeptides determining reovirus virulence

Genetic experiments have implicated two reovirus polypeptides in determining the tissue tropism and virulence pattern of reovirus infection. Reovirus serotypes 1 and 3 were originally isolated from the human gastrointestinal tract and the brains of mice retrospectively, and reovirus thus exhibits a wide tissue tropism.

The first experiments were designed to determine the genes responsible for reovirus virulence in mice after intracerebral injection. The different serotypes of reovirus differ in this regard - type 3 infects the neuronal cells of the brain and causes acute and fatal encephalitis, while type 1 infects

1.4.1.2.3 (Cont'd).

the ependymal cells lining the ventricular brain cavities and causes mild and non-fatal hydrocephalus (Margolis and Kilham, 1969; Margolis et al., 1971). In this case, the virulence of the virus is caused by its tissue tropism. By using reassortants between types 1 and 3, Weiner et al. (1977) showed that the neurovirulence pattern is associated solely with the S1 gene. The $\sigma 1$ protein encoded by gene S1 is the cell attachment protein in tissue culture (Lee et al., 1981b) so it presumably acts by determining the interaction of virus with cell receptors on neuronal or ependymal cells.

A different aspect of reovirus virulence has been shown to be determined by the M2 gene. On gastrointestinal infection, the infectivity of type 1 is much more resistant to proteases than type 3 and only type 1 can infect the gastrointestinal tract or survive after oral inoculation. Use of reassortant viruses has assigned this property to gene M2 (Rubin and Fields, 1980). M2 codes for $\mu 1$ and $\mu 1c$, which is the major outer capsid protein and determines resistance to proteases. The study was extended by injection of a reassortant containing an M2 gene of type 1 and an S1 gene of type 3 into the upper intestinal region of suckling mice. This reassortant

1.4.1.2.3 (Cont'd).

produced a fatal intracerebral infection resulting from initial multiplication in intestinal tissue and subsequent spread to the brain (Rubin and Fields, 1980).

Variants of serotype 3 with the same tissue tropism but decreased virulence have recently been identified (Hrady et al., 1982). The attenuation of neurovirulence was attributed to the M2 gene by genetic analysis, so M2 also seems to affect the virulence pattern of the virus after inoculation without altering the tissue tropism. M2 possibly determines how well the virus grows in the target cells.

1.4.1.2.4 Identification of the target antigen for cytolytic T lymphocytes

Spleen cells from animals infected with reovirus produce reovirus-specific cytolytic T lymphocytes (CTL). These lyse cells with the same H2 haplotype which also carry the appropriate viral antigen from the same serotype, although some cross reactivity against other virus strains is observed (Finberg et al., 1979). Use of reassortant viruses has shown that the S1 gene is predominant in determining CTL specificity. The cross-reactivity of CTL's could be due to CTL's directed against other virus cross-reactive antigens (as found with antisera).

1.4.1.2.4 (Cont'd.)

The reovirus $\sigma 1$ protein has also been associated with serotype specificity of delayed type hypersensitivity (Weiner et al., 1980) and specificity of immunological tolerance via suppressor T cells (Greene and Weiner, 1980). However, stimulation of suppressor T cells occurred after oral administration of a virus with a mutant $\mu 1c$ protein. Therefore, the M2 gene also plays a part in the pattern of immunological response after natural infection (Fields and Greene, 1982).

1.4.1.2.5 Determination of the reovirus antigen that interacts with cellular microtubules

Reovirus replication results in microtubules being coated with newly formed virus-specific proteins within viral factories, and reovirus type 1 binds much more tightly to microtubules than type 3. Use of reassortant viruses has shown that $\sigma 1$ determines the ability of reovirus to bind to microtubules.

1.4.1.2.6 Identification of the proteins determining inhibition of host cell macromolecular synthesis

The $\sigma 1$ protein has been shown to be responsible for reovirus inhibition of cellular DNA synthesis which takes place with reovirus type 3 but not with type 1

1.4.1.2.6 (Cont'd.)

(Sharpe and Fields, 1981). The $\sigma 3$ protein is responsible for inhibiting host cell RNA and protein synthesis (Ahmed and Fields, 1982). This may be part of the reason why $\sigma 3$ has a crucial role in attenuation of virus after high passage, and in the ability of D1 viruses to establish persistent infection (Fields and Greene, 1982).

1.4.2 Rotavirus genetics

The example of the contribution of reovirus genetics to understanding virus-host interactions has stimulated interest in rotavirus genetics, especially as rotaviruses are medically and economically more important than reovirus. As previously described (section 1.3.1), rotaviruses undergo genetic reassortment and are divisible into two subgroups and several different serotypes. However, all rotaviruses seem to cause the same gut pathology (section 1.2.3), so the study of reassortants may not resolve the molecular determinants of virulence as was done with reovirus. A possible alternative is to use host specificity as a marker since each serotype infects a different animal species but care is necessary in this regard because rotaviruses can infect other species as well as their own (McNulty, 1978).

The following sections describe the current progress in rotavirus genetics through the study of ts mutants and reassortants. The characterisation of both these groups is still at an early stage.

1.4.2.1 Production and characterisation of rotavirus *ts* mutants

Greenberg *et al.* (1981b) first produced *ts* mutants of a bovine rotavirus by chemical mutagenesis. Seven *ts* mutants were identified on the basis of efficiency of plaque formation and these were divided into four separate groups by reassortment. A larger group of 26 *ts* mutants of bovine rotavirus were isolated by Faulkner-Valle *et al.* (1982), twelve being *ts* by efficiency of plaque formation alone and fourteen being *ts* by efficiency of plaque formation and virus yield. These mutants were divided into five reassortment groups. The four groups of Greenberg *et al.* (1981b) have not been crossed with the five groups of Faulkner-Valle *et al.* (1982). The mutants of Faulkner-Valle *et al.* (1982) were analysed for RNA and protein synthesis at 39.5°C. Two of the groups were early mutants on the basis of temperature shift experiments and did not synthesise RNA or protein at 39.5°C. The other three groups produced both RNA and protein at 39.5°C, although one of them synthesised a reduced amount of both. No defect in any one RNA segment or polypeptide was noted. Ramig (1982) raised temperature-sensitive mutants of SA-11 rotavirus which fell into five reassortment groups. No complement was detected between these *ts* mutants, and mutants of all the mutant groups interfered with the growth of the wild type virus at permissive and non-permissive temperatures (Ramig, 1983).

1.4.2.2 Production and characterisation of genetic reassortants of rotavirus

Matsuno et al. (1980) produced a reassortant virus from calf and simian rotaviruses and showed that some RNA segments were derived from each parent. No function associated with either parent could be assigned to any one gene. Greenberg et al. (1981b) produced reassortant viruses between ts mutants of bovine rotavirus and each of two serotypes of noncultivable human rotavirus. They were selected in the presence of antiserum against bovine rotavirus and for growth in tissue culture at 39°C, thus ensuring at least one gene from each parent. Each reassortant was specifically neutralized by antiserum against the serotype of human rotavirus from which it was derived, and each was able to grow in tissue culture. In this way, the noncultivable human rotavirus was said to be 'rescued' while it retained the antigenic characteristics of human rotavirus. The RNA's were analysed and 3-5 segments were shown to be derived from the bovine rotavirus parent, but no specific function was assigned to any of them. This study was then extended by Kalica et al. (1981) who produced a variety of reassortants between bovine rotavirus ts mutants and human rotavirus type 2. Some of these were neutralized by human type 2 antiserum and others by bovine rotavirus antiserum, and they were also separately distinguishable by ELISA.

1.4.2.2 (Cont'd.)

The RNA segments of the reassortants were analysed and the authors concluded (a) that the gene coding for the antigen allowing neutralization was the human gene segment 9 or the calf segment 8 or 9 (these could not be separated) and (b) that the antigen determining viral subgroup was encoded by gene segment 6, i.e. the major inner shell capsid polypeptide VP6 (Dyall-Smith and Holmes, 1981). All the reassortants had gene segments 4 and 5 from bovine rotavirus, so one or both of these genes is presumed to enable growth of the reassortants in tissue culture. Gene 4 codes for the polypeptide VP3 which is cleaved in the presence of trypsin rendering the virus infectious and gene 5 encodes a non-structural polypeptide. Kalica et al. (1983) have now extended these genetic experiments and assigned both the haemagglutinin and the function of protease-enhanced plaque formation to the product of gene segment 4 (i.e. VP3).

MATERIALS AND METHODS

The materials used and their suppliers are as follows:

Chemicals

NP₄₀ - BDH Chemicals Ltd., Poole, Dorset, U.K.

Triton X-100 - BDH.

Protein A-Sepharose CL-4B - Pharmacia Fine Chemicals, Sweden.

Concanavalin A-Sepharose CL-4B - Pharmacia.

DEAE-cellulose - Bio-Rad Laboratories, Richmond, California, U.S.A.

Sephadex G50 and G25 - Sigma (London) Chemical Co., Poole, Dorset,
U.K.

Sodium deoxycholate - Sigma.

Dithiothreitol (DTT) - Sigma.

Coomassie brilliant blue - Sigma.

Oligo dT cellulose (type 3) - Collaborative Research Inc., Waltham,
Mass.

Arcton 113 (trichlorotrifluoroethane) - ICI, Mond Division.

Freunds Adjuvant - GIBCO, N.J.

Sagatal (pentobarbitone) - May and Baker Ltd., Dagenham, England.

Electrophoresis reagents

Acrylamide (specially purified for electrophoresis) and N-N'-methylene
bis acrylamide (specially pure) - BDH.

N,N,N',N' - tetramethylenediamine (TEMED) - Bio-Rad.

Radiolabelled compounds

All radiolabelled compounds were obtained from Amersham International,
Amersham, Bucks, U.K. The compounds had the following specific activities:

³⁵S-methionine: 600-1300 Ci/mmol.

Radiolabelled compounds (Cont'd)

[5'-³H] uridine: 25-30 Ci/mmol.

[5,6-³H] uridine 5' triphosphate: 40-60 Ci/mmol.

S-adenosyl-L-(methyl-³H) methionine (³H-SAM): 60-85 Ci/mmol.

¹²⁵Iodine: 350-600 Ci/mmol.

Cytidine 3,5-[5'-³²P] bisphosphate (³²pCp): 3000 Ci/mmol.

Guanosine-[α-³²P] triphosphate: 3000 Ci/mmol.

Enzymes and Substrates

Sigma supplied nucleoside triphosphates (UTP, CTP, GTP, ATP and di-P-ATP), amino acids, phosphoenol pyruvate (PEP), pyruvate kinase, creatine phosphokinase, micrococcal nuclease, nucleases T₁, S1 and A, haemin, S-adenosyl methionine, trypsin (2 x recrystallised) and S.aureus V8 protease.

Lactoperoxidase came from Boehringer Corporation Ltd., Lewes, U.K., T₄ RNA ligase and S-adenosyl homocysteine from PL Biochemicals Inc., Milwaukee, Wis. and tunicamycin was a gift from Dr. Hamill, Eli Lilly, U.S.A.

Scintillants

Toluene-PP0 was made from 12.5gPP0 in 2.5 litres of toluene (5g/l).

Triton-toluene scintillant contained 4gPP0, 0.05g POPOP, 333 ml

Triton X-100, 666 ml toluene.

Scintillant-solubiliser also contained NCS solubiliser.

Film

Kodak X-Omat H light sensitive X-ray film was supplied by Kodak. Fuji RX X-ray came from Fuji Photo Film Co. Ltd., Tokyo. Other films and papers for light photography were obtained from Kodak.

Antisera and Immunoglobulins

Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-bovine gamma globulin and swine anti-guinea pig gamma globulin were supplied by Nordic Immunologicals Ltd., Maidenhead, Berks. Rotavirus antisera were kindly provided by Dr. J. Bridger, I.R.A.D., Compton, U.K., Dr. Snodgrass, Moredun Institute, Edinburgh and Dr. Bachmann, University of Munich. (The antisera are described in Table 1).

Media

Tissue culture media were all purchased from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland. Foetal calf serum (FCS) was obtained from GIBCO, Grand Island, N.Y., U.S.A. Noble Agar, bacterial Penassay broth, yeast extract and vitamin assay casamino acids came from Difco Laboratories, Detroit, Michigan, U.S.A.

Cells

BSC-1 African green monkey kidney cells were a kind gift from Dr. C. Pringle, MRC Institute of Virology, Glasgow, Scotland, and were cultured in Glasgow's modified Eagle's minimal essential medium (GMEM) containing 5% (v/v) foetal calf serum, glutamine and crystamycin (Glaxo) or stored in liquid nitrogen (see below).

TABLE 1 - Description of rotavirus antisera used in
immunoprecipitation

- C1 = G203 (1973). Convalescent calf serum raised in a gnotobiotic calf by one oral inoculation of the Compton strain of the tissue culture adapted calf rotavirus. The serum was shown to be free of antibody to bovine diarrhea virus by neutralization. Neutralization titre of G203 was approximately 6,000, or 40 by the immunofluorescent method. Provided by Dr. J. Bridger, IRAD, Compton.
- C2 = C1 which was aliquoted in 1976.
- C3 = Pooled calf serum; serum from several calves which had not been inoculated with rotavirus and therefore had an undetermined natural anti-rotavirus antibody content.
- C4 = Hyperimmune calf serum to calf rotavirus strain V10. The titre of the antisera was approximately 12,500 by ELISA. Provided by Dr. P. Bachman, University of Munich.
- C5 = Commercial newborn calf serum (not immunised with rotavirus). Provided by Flow Laboratories.
- C6 = Rabbit anti-calf rotavirus serum against bovine serotype 1 rotavirus which had been grown on CK cells. The serum was raised by hyperimmunisation. Other details of the serum were not available. Provided by Dr. Snodgrass, Moredun Institute, Edinburgh.

P1 = Isol 42A (1980). Antiserum raised by one oral inoculation of a gnotobiotic piglet with a 0.45 μ filtrate of a field isolate of porcine rotavirus (SW 20/21) which had been passed 8 times through gnotobiotic piglets. The serum was not tested for titre or cross-reactions. Provided by Dr. Bridger.

P2 = Convalescent antiserum produced against porcine rotavirus in a gnotobiotic piglet, possibly by hyperimmunisation. Details of the serum were not available. Provided by Dr. Snodgrass.

H1 = Hyperimmune rabbit serum raised against both subgroups of human rotavirus, concentrated from faecal extracts. Random injections of either subgroup were given in at least 8 inoculations over 3 years. The titre was at least 400 00 by indirect ELISA and 10,000 by immunofluorescence against calf rotavirus infected cells. Provided by Mr. G. Beards, East Birmingham Hospital, Birmingham.

H2 = Convalescent serum raised in a gnotobiotic lamb against human rotavirus, possibly by hyperimmunisation. Further details of this serum were not available. Provided by Dr. Snodgrass.

L1 = Convalescent serum raised in a gnotobiotic lamb against lamb rotavirus. Details of this serum were not available. Provided by Dr. Snodgrass.

R1 = Normal rabbit serum from 6 rabbits in our animal house,
none of which had ever been immunised.

Ho = Commercial horse serum from Flow Laboratories.

F = Foetal calf serum from Flow Laboratories.

Virus

The U.K. strain of calf rotavirus had been adapted to tissue culture by passaging 28 times in primary calf kidney cells by Dr. J. Bridger, I.R.A.D., Compton and was provided after a further 19 passages in LLCMK₂ cells by Dr. M. Thouless, Regional Virus Laboratory, East Birmingham Hospital, Birmingham and plaque purification three times by Dr. G. Faulkner-Valle, Warwick University. The tissue culture adapted strain was also used to infect a gnotobiotic calf and the calf's faecal material was kindly supplied by Dr. J. Bridger. OSU porcine rotavirus was supplied by Professor H. Bohl.

Cell freezing

Cell suspensions were centrifuged at 800 rpm for 10 mins, and resuspended in GMEM with 20% FCS to a cell concentration of 4×10^7 cells/ml. An equal volume of GMEM containing 20% FCS and 16% DMSO was added. Optimal recovery of cells was obtained when they were frozen at less than 1°C per minute: 2 ml aliquots were held on ice for 30 mins, then placed at -20°C for 2 hrs, followed by -70°C overnight with occasional mixing until they were frozen. The cells were kept at the bottom of the -70°C freezer for 2-3 days and a vial was revived. If the cells were still viable, the remainder were stored indefinitely in liquid nitrogen.

Cells were revived by rapidly thawing at 37°C and seeding into small vials containing warmed GMEM with 5% FCS. The medium was changed after approximately 3h when the cells had settled and again after 8-12h.

Virus growth

Virus was passaged in confluent monolayers of BSC-1 cells that had been washed x 3 in serum-free medium. The virus was inoculated at a multiplicity of less than 0.1 p.f.u./cell and serum free medium containing 10µg/ml trypsin was added. After 48h at 37°C, by which time all the cells were floating in the medium (partly due to trypsin action), medium and cells were harvested, aliquoted and stored at -70°C.

Virus plaque assay

Virus was titrated on confluent monolayers of BSC-1 cells in 30 mm Petri dishes. Dilutions of virus were inoculated onto serum-free cells for 1h and unadsorbed virus was washed off x3 with serum-free medium. The cells were overlaid with serum-free medium + crystamycin in 0.9% agar and incubated for 3 days at 37°C. Trypsin was omitted as it sometimes caused the cells to detach from the dish. The cells were fixed in formal saline (3.7% formaldehyde in 0.5% NaCl, 1.5% Na₂SO₄) for 30 min. The agar overlay was removed and the cells were stained with 0.1% crystal violet in 20% ethanol. Plaques appear as areas which stain more intensely than non-infected cells and are termed foci (Faulkner-Valle et al., 1982). These foci appear as heaped necrotic cells which have not lysed. Later in infection the foci lyse into clear plaques, but due to the need for a serum-free overlay medium, cells did not always survive for this length of time.

Plaque reduction assay

Neutralization of calf rotavirus by guinea pig monospecific antisera or calf anti-calf rotavirus antiserum was determined by a standard plaque reduction assay. Antiserum was diluted from 1/20 in four-fold steps in phosphate buffered saline (PBS) and mixed with an equal volume of virus containing a small number of pfu's. The virus and antiserum were incubated for 2h at 25°C and the residual infectious virus titrated, as well as controls of virus with pre-immune serum and without serum incubated in the same way. The plaque assay has been described above. Care was necessary to wash the virus-serum inoculum off the cells, so that controls without serum would not have disproportionately high titres in relation to the test, due to serum inactivating trypsin in the virus inoculum.

Virus purification

Reoviruses are cell associated and are routinely purified by fluorocarbon extraction of infected cells, while all the cell medium is discarded (Smith et al., 1969). Rotavirus is either less cell associated or the trypsin present during its growth weakens the integrity of the cells as some virus has been found in the medium, so purification of both cells and the medium is carried out. EM examination has shown some rotaviruses in the medium to be surrounded by cell membrane and these are released by fluorocarbon extraction.

Cells were pelleted at 5,000 x g for 30 min and virus pelleted from the supernatants at 75,000 x g in an MSE 6 x 300 ml

Virus purification (Cont'd)

angle rotor for 3h. Virus was extracted from the combined pellets by homogenisation with Arcton (ICI) for 3 min and separation of the aqueous and organic phases by low speed centrifugation. The organic layer was re-extracted with resuspension buffer [50 mM Tris-HCl pH8.0, 10 mM NaCl, 2mM CaCl_2 , 1.5 mM β -mercaptoethanol (BME)] twice, and all aqueous phases were then centrifuged at 100,000xg in an MSE 3 x 70 ml rotor to pellet the virus. The pelleted virus fractions were then layered onto caesium chloride (density 1.37g/cm^3) in 10 ml centrifuge tubes and centrifuged at 100,000xg for 18h in a 10 x 10 ml angle rotor so that a density gradient was formed. Single shelled particles formed a band at 1.38g/cm^3 and double shelled particles at 1.36g/cm^3 . These bands are clearly separated (fig. 1). Centrifugation on preformed CsCl gradients for 2h at 100,000 xg did not separate the two virus bands as clearly. Fractions from the gradients were collected from the top using an Isco fraction collector and the refractive indices were measured. Virus containing fractions were diluted, pelleted and resuspended in 50mM Tris-HCl pH8.0, 2 mM CaCl_2 .

Single shelled particles were also prepared by treatment of purified virions with 1.5 mM EDTA for 30 min at 37°C and collected by centrifugation through 45% sucrose in 50 mM Tris pH8.0 at 100,000 xg for 90 min. These single shelled particles were free from contaminating double shelled particles

Virus purification (Cont'd)

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FIG 1

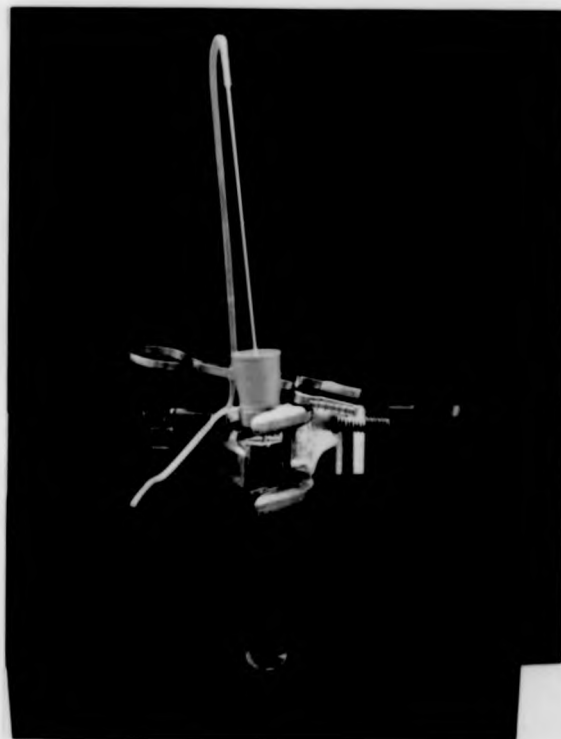
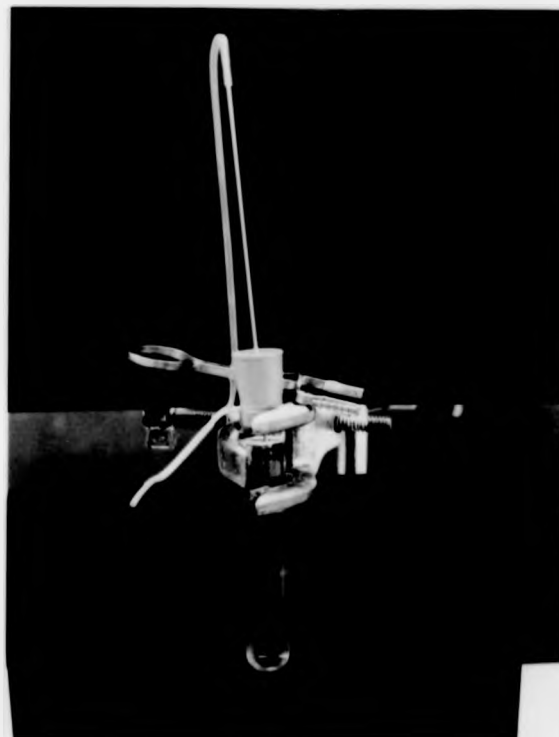


FIG 1



Virus purification (Cont'd)

and could be analysed by PAGE or used as a pure source of single shelled particles for IEM.

Radiolabelling of virus induced polypeptides in infected cells

Virus proteins synthesised in infected cells were analysed by pulse labelling of the infected cells at various times after infection, particularly during peak protein synthesis between 6-8h after infection. High multiplicities of infection (M.O.I. = 10) were necessary to obtain strongly radiolabelled virus proteins and to shut off host protein synthesis. However, for radiolabelled cell extracts for immune precipitation 5 p.f.u./cell was used as at this multiplicity there was still some host cell protein synthesis which was a useful control on the specificity of the precipitation.

Confluent monolayers of BSC-1 cells in 30mm Petri dishes were washed in serum-free medium. The virus was inoculated onto the cells for 1h, was then removed and replaced with medium containing 1% of the normal amount of methionine. For pulse labelling (usually at 6h.p.i.) the medium was removed and replaced by 100 μ l PBS containing 50 μ Ci 35 S methionine. After labelling for 30 min at 37°C, the cells were washed, scraped into 50 mM Tris pH7.4 and stored at -20°C. For some experiments, cells were infected in the presence of tunicamycin, which inhibits glycosylation (Schwarz *et al.*, 1976). Medium containing 5 μ g/ml tunicamycin was used throughout infection

Radiolabelling of virus induced polypeptides in infected cells (Cont'd)
and the pulse-labelling was also done in the presence of
5µg/ml tunicamycin.

³⁵S methionine labelled virus was also prepared so that
its polypeptide pattern could be compared with that seen in
infected cells. 2.5ℓ roller bottles containing methionine-
starved BSC-1 cells were infected at an m.o.i. of 1 p.f.u./cell
and incubated for 48h at 37°C in 25 ml medium containing 10%
of the normal concentration of methionine, 20 µCi/ul
³⁵S-methionine and 10 µg/ml trypsin. The virus was harvested
and purified as already described. Non-radiolabelled purified
rotavirus was radiolabelled in vitro with ¹²⁵I by the
chloramine T method of Syvanen et al. (1973). As the iodine
is reduced and neutralized prior to mixing with the virus,
damage to the protein is minimised. A mixture of 15 µl
0.1N H₂SO₄ and 15 µl 1.5 mM chloramine T was added to
15 µl 1mM KI and 250 µCi ¹²⁵I. After 30 sec. incubation,
the mixture was neutralized with 15 µl 1.0M KH₂PO₄. The
virus was then added for 2 min and the reaction terminated
with 10 µl of 1.0M BME. The virus was pelleted at
100,000 xg for 90 min through 45% sucrose to separate it from
free ¹²⁵I.

Preparation of cytoplasmic extracts

Cells were suspended in hypotonic TNE buffer (10 mM Tris-HCl
pH7.3, 10 mM NaCl, 10 mM EDTA) and allowed to swell for
10 min on ice. Nonidet P₄₀ (NP₄₀) was added to a final
concentration of 0.5% v/v for 10 min on ice and then the

Preparation of cytoplasmic extracts (Cont'd.)

cells were further disrupted in a Dounce homogeniser with 20 strokes, centrifuged at 14,000 xg for 1 min and the supernatants (the cytoplasmic extract) stored at -20°C.

TCA precipitation and estimation of radioactivity in polypeptides

Aliquots of radiolabelled polypeptides were dried onto filter papers and put into cold 10% TCA for 10 min. They were then boiled in 5% TCA for 5 min, washed twice in ethanol and dried. Filters were placed in scintillation vials with 4 ml toluene-PP0 and the radioactivity counted in a Packard scintillation counter.

Preparation of formalin fixed *Staphylococcus aureus* for use in immune precipitation.

The Cowan 1 strain of *S.aureus* which has protein A on its surface was prepared as described by Kessler (1975). The Cowan 1 strain was provided by Dr. C.Pringle, MRC Institute of Virology, Glasgow and grown in Penassay broth supplemented with 2.5 g/l yeast extract and 5g/l casamino acids. A starter culture of 10 ml was grown overnight at 37°C and then transferred into 2l broth in Erlenmeyer flasks and grown for 24h at 37°C. The bacteria were collected by centrifugation at 8,000 xg for 10 min and washed twice in PBS containing 0.05% (w/v) sodium azide. The bacteria were then suspended at a concentration of 10% (w/v) in PBS-azide and stirred for 1½h at 23°C in the presence of 1.5% formalin, washed and resuspended at 10% in PBS-azide. The bacteria were then added to a large Erlenmeyer flask to a depth of less than

Preparation of formalin fixed Staphylococcus aureus for use in
immune precipitation (Cont'd).

1.5 cm, stirred for 5 min at 80°C and cooled rapidly in iced water. The cells were washed twice more in PBS-azide, suspended at 10% (v/v) in PBS-azide, aliquoted and stored at -70°C.

Immediately before use in immune precipitation, bacteria were pelleted by centrifugation at 14,000 xg for 1 min and incubated in 100 mM Tris-HCl pH8.2, 100 mM KCl, 5 mM MgCl₂, 1% Triton X-100 for 15 min at room temperature. The bacteria were pelleted, washed once and resuspended at a 10% suspension in the same buffer with 0.5 mg/ml bovine serum albumin.

Immune precipitation

Rotavirus polypeptides were immunoprecipitated from infected cell extracts with anti-rotavirus serum using a modification of the method of Kessler (1975), which uses a higher concentration of detergent to increase the specificity of precipitation and centrifugation of the cell extracts before use to remove aggregated material.

Cytoplasmic extracts were prepared as described above; 20 µl were diluted into 500 µl with freshly prepared "high" detergent buffer [100 mM Tris-HCl pH8.2, 100 mM KCl, 5 mM MgCl₂, 1% Triton X-100, 1% (w/v) sodium deoxycholate, 0.5% (w/v) SDS]. This was then centrifuged at 14,000 xg for 3 min to remove aggregates. The supernatant was incubated with 10 µl undiluted

Immune precipitation (Cont'd.)

antiserum for 1h at room temperature, and then 25 μ l formalin fixed S.aureus (10% v/v) in detergent buffer was added and the mixture stirred for 18h at 4°C. The precipitate was collected by centrifugation and washed once in detergent buffer. The precipitated proteins were solubilized with 2% (w/v) SDS and 5% BME at 100°C for 5 min before analysis by PAGE.

The specificity of immune precipitation was further increased by denaturing the proteins in the infected cell extract in 2% SDS and 5% BME at 100°C for 2 min before dilution in detergent buffer. Apparently this separated protein complexes into polypeptides, allowing each to react separately in the immune precipitation reaction.

Protein A-Sepharose chromatography

Immunoglobulins were purified from serum on columns of protein A-Sepharose. Protein A-Sepharose CL-4B was washed with PBS and stirred with antiserum (250 μ l protein A-Sepharose to 1 ml antiserum) overnight at 4°C. A 1 ml syringe was then packed with the mixture and the column washed with PBS until the optical density of the eluate was below 0.1 OD units at 280 nm. The column was then washed with 2 ml PBS containing 1% NP₄₀ followed by further washing in PBS until the eluate was less than 0.1 OD units. Immunoglobulin bound to the protein A was then eluted with 0.15M NaCl, 0.6% acetic acid pH 3.0. Fractions were assayed

Protein A-Sepharose chromatography (Cont'd.)

spectrophotometrically and peak fractions were pooled and neutralized with KOH before storage in aliquots at -20°C.

^{125}I labelling of immunoglobulins

Lactoperoxidase-catalysed iodination was performed using a modification of the method described by Marchalonis (1969). 100 µg immunoglobulin was diluted into 1 ml PBS containing 100 µCi ^{125}I and 10 µl 10^{-5}M KI and 10 µl lactoperoxidase were added. 5 aliquots of 5 µl 0.5 mM H_2O_2 were added at 30 sec intervals and, after a further 30 sec, the reaction was stopped with 100 µl 1% sodium azide. Protein was separated from free ^{125}I by chromatography on a column of G-25 sephadex in PBS.

Immunofluorescence

Indirect immunofluorescence of calf rotavirus infected cells was performed by the method of Thouless et al. (1977). Subconfluent cell monolayers on glass coverslips were infected at an m.o.i. of approximately 5 p.f.u./cell. After adsorption, the coverslips were incubated with serum-free medium at 37°C for 12h and then washed with PBS and fixed with methanol (which had been kept at -70°C) for 20 min at -20°C. The coverslips were dried and stored at -20°C or washed with PBS prior to incubation with serum. Dilutions of anti-rotavirus serum were added onto the coverslips (50 µl per coverslip) and incubated for 1h at 37°C in a moist atmosphere. The cells were then washed twice with

Immunofluorescence (Cont'd).

PBS at 37°C for 20 min each time, and then incubated with 50 µl per coverslip of FITC-conjugated antiserum diluted 1/20 (rabbit anti-bovine IgG or swine anti-guinea pig IgG). This was incubated for 1h at 37°C followed by two washes in PBS for 20 min each. The cells were then counterstained with Evan's blue diluted 1/150,000 for 5 min, dipped in distilled water and dried. Coverslips were mounted in glycerol-saline (50% glycerol, 150 mM NaCl) and observed under a Reichart Binolux II UV microscope for fluorescence. Controls of uninfected cells stained as above or infected cells incubated with pre-immune serum instead of rotavirus antiserum were used.

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels were set up using the Laemmli (1970) discontinuous buffer system. 10-30% (w/v) linear polyacrylamide slab gels (20 x 20 cm) were prepared as described by Cook et al. (1979). 10% solutions contained 4.1 ml, 60% acrylamide plus 0.282% bisacrylamide solution; 9.3 ml 1M Tris-HCl pH8.6; 0.25 ml 10% SDS; 2.5 ml 80% glycerol and 8.8 ml water. The 30% acrylamide solution varied in the amount of acrylamide (12.5 ml) and water (0.4 ml). Before pouring the gels, 33 µl TEMED was added to each solution, as well as 16 µl 10% ammonium persulphate (APS) to the 30% acrylamide and 33 µl 10% APS to the 10% acrylamide, forming a negative persulphate gradient. Gradients were poured from a two chamber gradient maker, stopped when the gel was about 5 cm from the top of the notched plate, and allowed to

Polyacrylamide gel electrophoresis (PAGE) (Cont'd).

polymerise under water-saturated butanol. A stacking gel of 1.5 ml 30% acrylamide, 0.8% bis-acrylamide; 6 ml H₂O; 2.5 ml 0.5M Tris-HCl pH6.8, 0.4% SDS; 33 µl TEMED and 30 µl 10% APS was poured on top of the separating gel. Samples in 10 mM Tris-HCl pH7.5, 2% SDS, 5% BME and 5% glycerol were heated at 100°C for 2 min and 25 µl of each loaded onto the gels. After electrophoresis at 14 mA for 16h the gels were usually fluorographed (Laskey and Mills, 1975) or dried and exposed to X-ray film. For fluorography, the gels were gently shaken in two changes of DMSO for 30 min each and then in DMSO containing 22.2% PPO for 3h. After washing for 30 min in running water the gels were dried under suction and exposed to Kodak X-Omat H film. Some gels were stained with 1.25% Coomassie brilliant blue in 10% acetic acid, 50% methanol for 1h and destained in 10% acetic acid, 50% methanol.

RNA gels

dsRNA was electrophoresed on 6.5% non-gradient polyacrylamide gels containing 0.174% bis-acrylamide using the Laemmli system previously described.

Excision of bands from gels and scintillation counting

Exposed and developed film was aligned with the dried gel using radioactive ink markers and the positions of the polypeptides were marked out. These were excised and cut into small pieces

Excision of bands from gels and scintillation counting (Cont'd)

and incubated in H₂O₂ at 80°C for 3h or in scintillant/solubiliser for 48h at 37°C in sealed glass vials. The samples were then chilled and scintillant was added to the H₂O₂ samples and the radioactivity measured in a scintillation counter.

Peptide mapping

Polypeptides were subjected to partial proteolysis, and the resulting peptides analysed by single dimension electrophoresis using a modification of the method of Cleveland et al. (1977) as described by McCrae and Joklik (1978). Polypeptides from SDS polyacrylamide gels were excised, cut into small pieces and allowed to swell in stacking gel buffer (0.125M Tris-HCl 0.1% SDS pH 6.8) for 10 min at room temperature. The gel slices were mashed, separated into aliquots centrifuge tubes and S.aureus V8 protease was added at 5 µg/ml and 50 µg/ml respectively for 30 min at 37°C. Control aliquots without V8 were kept on ice. The tubes were then pierced at the bottom, fitted into larger centrifuge tubes and centrifuged for 5 min at 2,000 rpm, so that all peptides that had come out of the acrylamide went through into the larger tube. Aliquots from each tube were counted in an attempt to equalize the loading on the gels, and the rest was made 2% for SDS and 5% for BME, boiled for 2 min and analysed by PAGE.

Maintenance of guinea pigs free from rotavirus

Following the detection of rotavirus antibody in the serum of rabbits in the animal house, the guinea pigs were isolated to avoid cross infection. A separate room was sealed, fumigated and maintained in a reversed airflow to exclude air from inside the building. Each animal was kept in a separate cage. Cages, food, hay, water and equipment were all sterilised before use. The water was supplemented with 1.25 g/l ascorbic acid. Young female guinea pigs of Duncan-Hartley strain (Olac 1976 Ltd., Bicester, Oxon) weighing 450-550g each were used, and their serum was screened for rotavirus antibody before immunisation.

Preparation of individual rotavirus polypeptides and immunisation of guinea pigs.

Proteins from approximately 1 mg of purified virus were separated by PAGE. Radioactive virus was added to locate the protein (see above). Proteins were excised from the dried gels, cut into small pieces and swollen in Tris-NaCl pH7.4 for 18h at 4°C. The gel was mashed by syringing through 13g, 15g and 17g needles and homogenised with Freund's complete adjuvant for the first injection or Freund's incomplete adjuvant thereafter. Antigen was injected subcutaneously at 3 week intervals until an antibody response was detected by immunofluorescence or immunoprecipitation. Test bleeds were taken from the ear, and the final bleeds were taken from the heart. Blood was allowed to clot at 4°C overnight and serum recovered by centrifugation.

Transcription of rotavirus mRNA in vitro

Transcription by partly disrupted rotavirus particles was achieved in vitro in the presence of all four nucleoside triphosphates, an energy source and magnesium ions. The RNA transcriptase present in purified virions was activated by incubation at 37°C for 20 min in transcription mix without magnesium and containing 1.5mM EDTA. The EDTA has been shown to remove the viral outer shell (Cohen, 1977). Following this activation step, Mg^{++} was added to a final concentration of 9mM and this stimulated the incorporation of [3H] UTP into acid insoluble material.

The transcription mix used contained 4 mM GTP, 4 mM CTP, 5.5 mM ATP, 2 mM UTP, 50 μ Cl/ml 3H -UTP (40 Ci/mmol), 1.6 mM phospho-enol-pyruvate (PEP), 45 units/ml pyruvate kinase, 0.2 mM s-adenosyl methionine, 0.5 units/ml inorganic pyrophosphatase, 30 μ g/ml bentonite and 100 mM Tris-HCl buffer pH8.0. 3H -methyl labelled transcripts were obtained using a reaction mixture in which the unlabelled s-adenosyl methionine (SAM) was replaced with 3H -methyl labelled SAM at a final concentration of 50 μ Cl/ml (15 Ci/mmol) and 3H -UTP was omitted.

Transcription mixtures were incubated for up to 8h at 37°C with continuous agitation. Virus (single shelled) particles were then removed by centrifugation for 20 min at 14,000 xg and resuspended in fresh transcription reaction mixture for further rounds of transcription. The supernatant was extracted with

Transcription of rotavirus mRNA in vitro (Cont'd)

phenol, and then the single stranded RNA transcripts were precipitated by adding lithium chloride (LiCl) to a final concentration of 2M and incubating at 4°C for 16 hrs. Precipitates were collected by centrifugation at 14,000 xg for 2 min and the precipitate was dissolved in 1mM EDTA and reprecipitated in 2½ volumes of ethanol plus 0.15M NaCl at -20°C. The ethanol precipitates were washed with 80% ethanol and 100% ethanol, dried by vacuum dessication, resuspended in 1mM EDTA and stored at -20°C.

Reovirus mRNA was transcribed in the same reaction conditions with 13mM Mg⁺⁺ and the transcripts were purified as described above. Reovirus particles were activated by pretreatment with 50 µg/ml chymotrypsin at 37°C for 65 min, which removes the reovirus outer shell.

Sucrose gradient analysis of in vitro transcripts

15% sucrose and 30% sucrose solutions were prepared in TLES (50 mM Tris-HCl pH7.5, 100mM LiCl, 1mM EDTA, 0.1% SDS). Linear 15-30% sucrose gradients were prepared by running the lighter solution into the heavier using a gradient maker. The transcripts in aqueous solution were layered onto the gradients and centrifuged at 30,000 rpm in an MSE 6 x 14 ml rotor for 30h at 20°C. Reovirus transcripts were optimally fractionated after 18h in the same conditions. Gradients were harvested in

Sucrose gradient analysis of in vitro transcripts (Cont'd).

an ISCO gradient collector. Aliquots from each fraction were dotted onto filter papers, washed x 2 in cold 5% TCA, once in ethanol, dried and counted in Toluene-PP0 in a Packard scintillation counter.

Extraction of dsRNA

Purified virus suspension in 50 mM Tris pH8.0 was made up to 2% SDS, and an equal volume of water-saturated phenol was added and shaken for 10 min at room temperature. The phenolic and aqueous phases were separated by centrifugation and the procedure repeated twice. Phenol was then removed from the aqueous phase with ether, and the ether blown off in a nitrogen stream. The RNA was made 0.375 M for NaCl and precipitated with 2½ volumes of ethanol at -20°C for 16h. The RNA was washed and stored as described above.

3' End labelling of mRNA and dsRNA.

RNA was 3' end labelled with 32 pCp and T_4 RNA ligase as described by England and Uhlenbeck (1978). RNA was added to an end labelling reaction mixture containing 150 μ Cl/ml 32 pCp, 50 mM HEPES pH8.3, 10 mM $MgCl_2$, 3 mM DTT, 3 μ M ATP, 6% DMSO, 10% glycerol and 200 units/ml T_4 RNA ligase. After 19h at 4°C followed by 10 min at 37°C, the reaction was terminated by adding an equal volume of double strength column buffer. The unincorporated 32 pCp was separated from the labelled RNA on Sephadex G50 columns with column buffer of 50 mM Tris pH8.0, 0.5M NaCl, 0.1M SDS.

In vitro translation

a) Preparation of rabbit reticulocyte lysates.

Reticulocytes were obtained from the blood of young rabbits made anaemic by injection with 1 ml of 100 µg/ml vitamin B12, 1 mg/ml folic acid in 0.15M NaCl pH7.0 followed by 0.6 ml of 2.5% acetylphenylhydrazine (Hunt and Jackson, 1974). Phenylhydrazine was injected daily for 5 days and the rabbits were then bled on the 7th day after a day's rest. The animals were anaesthetised with 1 ml Sagatal into the ear vein and bled by cardiac puncture, which yielded 50-80 ml from each rabbit. All syringes and containers were treated with 1,000 units/ml heparin to prevent clotting. For the following procedures the blood was kept on ice: blood cells were centrifuged at 2,000 rpm for 20 min, washed x 2 in cold buffer containing 0.14M KCl, 50 mM NaCl, 5mM MgCl₂ and the supernatant discarded. The packed cell volume was estimated and the cells lysed by adding an equal volume of distilled H₂O. Cell debris was removed by centrifugation at 8,000 rpm and the lysate was aliquoted into 2 ml volumes and stored in liquid nitrogen.

b) Translation of mRNA in reticulocyte lysates.

The translation system in which mRNA was translated contained 100 µM each of 19 amino acids (excluding methionine), 1 mM ATP, 200 µM GTP, 14mM creatine phosphate, 5.5 units creatine phosphokinase, 80 µg/ml mouse liver tRNA, 25 µCi ³⁵S-methionine,

b) Translation of mRNA in reticulocyte lysates (Cont'd.)

2 mM magnesium acetate, 120 mM KCl, 10mM Tris pH 7.4 and 50 μ l lysate in a final reaction volume of 100 μ l. 2-5 μ l mRNA was added to a final concentration of 25-50 μ g/ml. For convenience, amino acids were made up in solution containing 2.5 mM of each and stored at -20°C . The energy source containing 20 mM ATP, 4 mM GTP, 280 mM creatine phosphate and 110 units creatine phosphokinase was also made in advance and stored at -20°C .

The translation mixture was prepared on ice and, once the RNA had been added, was incubated at 30°C for 60 min. Aliquots were removed at timed periods onto filter papers which were TCA precipitated and the radioactivity estimated as described above.

Oligo dT cellulose column fractionation of mRNA

Oligo dT cellulose binds poly A which is often present at the 3' end of mRNA's. The in vitro transcripts were loaded onto a column of oligo dT cellulose in 50 mM Tris pH7.4, 0.4 M KCl and washed with this buffer until all unadsorbed material had washed through. Poly A containing mRNA bound to the column was then eluted with 50 mM Tris pH7.4.

DEAE cellulose chromatography of digested mRNA

mRNA was either digested completely in 0.3M KCl for 18h at 37°C or partly digested in 50 mM sodium carbonate/sodium bicarbonate pH 9.0 (50 mM sodium bicarbonate buffered to pH9.0 with 50 mM sodium carbonate) for 10 min at 90°C. The RNA was then neutralized and diluted in 5 mM Tris pH7.8 and run into a DEAE cellulose column. The nucleotides of increasing net negative charge were separated by elution from the column with a buffer salt gradient of 0.02-0.3M NaCl in 7M urea, 5 mM Tris-HCl pH7.8.

TCA precipitation of RNA

Radiolabelled RNA was spotted onto filter papers, dried and washed twice with ice cold 5% TCA for 10 min each time. The filters were then washed twice in ethanol, allowed to dry and the radioactivity counted in 0.4% PPO in toluene in a Packard scintillation counter.

RNA hybridisation

3' end labelled mRNA was hybridised to dsRNA to facilitate the fractionation of mRNA on polyacrylamide gels, as well as to test the authenticity of mRNA produced in vitro. 100 µl mRNA in 1 mM EDTA was mixed with dsRNA, and the solution was made 90% for DMSO and heated at 45°C for 20 min to separate the strands of dsRNA. 414 µl of hybridisation buffer (0.102M NaCl, 0.034M Tris-HCl pH7.4, 6.59mM EDTA) was added, and the RNA's were allowed to hybridise under these low salt conditions for

RNA hybridisation (Cont'd)

48h at 37°C (Ito and Joklik, 1972). Single stranded RNA was digested with nuclease (200 units/ml T₁ nuclease + 50 µg/ml pancreatic nuclease A) in 0.3M NaCl, 0.03M Na-citrate pH7.4 for 30 min at 37°C and the proportion of nuclease resistant radioactivity was estimated. dsRNA was analysed by PAGE as previously described.

Digestion of RNA with S1 nuclease

5µg dsRNA or mRNA was digested with 1,000 units/ml S1 nuclease in 50 mM sodium acetate pH4.5, 5 mM-zinc chloride at 37°C for 1h. Nuclease resistance was estimated by comparing the TCA precipitable c.p.m. with those present in samples incubated without addition of S1 nuclease.

CHAPTER 2

THEORY OF THE PROPERTIES OF THE POLYMER SOLUTION

The theory of the properties of the polymer solution is based on the assumption that the polymer chains are represented by a random coil. The theory of the properties of the polymer solution is based on the assumption that the polymer chains are represented by a random coil. The theory of the properties of the polymer solution is based on the assumption that the polymer chains are represented by a random coil.

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CHAPTER 3

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CHAPTER 3

Characterisation of the products of the rotavirus RNA polymerase

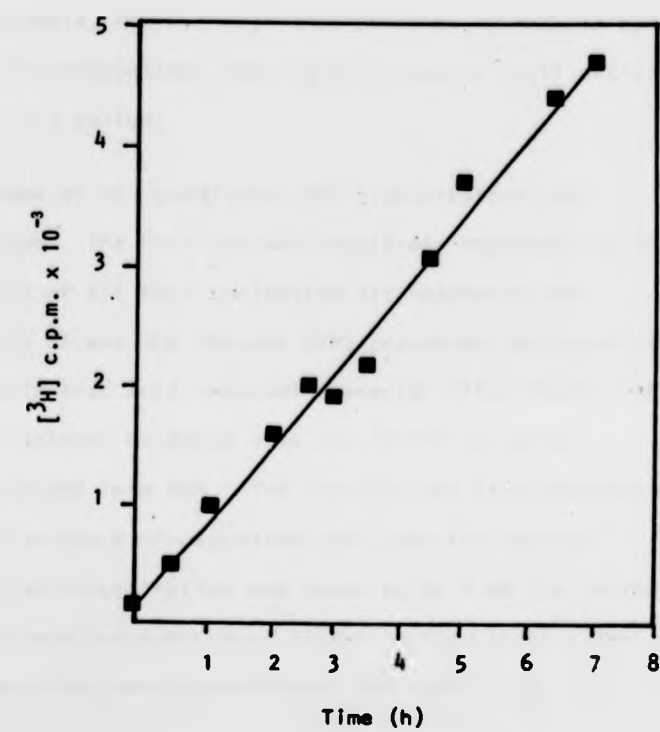
RNA polymerases of the reovirus family have been discussed in the introduction. At the commencement of this study, rotavirus RNA polymerases had been identified (Cohen, 1977; Hruska *et al.*, 1978) but the products of the polymerase reaction had not been studied. This section therefore describes the characterisation of these products.

3.1 Characterisation of the rotavirus RNA polymerase activity

Cohen (1977) showed that purified rotaviruses would not transcribe mRNA *in vitro* unless activated by pretreatment of the virus in 1mM EDTA for 20 min at 37°C. To avoid a separate pretreatment step, we found that the endogenous RNA polymerase activity could be activated by incubating purified rotavirus in transcription mix without magnesium but containing 1.5mM EDTA for 20 min at 37°C. After this period magnesium was added to the transcription mix and the RNA polymerase activity was assayed by measuring the incorporation of ^3H -UTP into acid insoluble material. A time course of RNA synthesis in this reaction is shown in Fig.1. There was a linear increase in the amount of product with time for at least 8h at 37°C. As had previously been reported for reovirus (Skehel and Joklik, 1969), polymerisation could be reactivated after transcription ceased if the virus was pelleted and

Fig.1 Time course of rotavirus in vitro transcription.

Purified rotavirus was incubated in standard reaction conditions as described in materials and methods. Samples were taken at intervals and counted for TCA insoluble radioactivity.



3.1 (Cont'd.)

resuspended in fresh transcription mix. Fig.2 shows the results of four rounds of transcription from a single virus sample, and although incorporation is reduced by the third incubation, the transcriptase is still active after this period.

Some of the conditions for transcription were optimised. The reaction was completely dependent on the presence of all four nucleoside triphosphates and omission of any one (except UTP) prevented incorporation of ^3H -UTP into acid insoluble material [fig. 3(a)]. This is preliminary evidence that the ^3H -UTP is being incorporated into RNA. The reaction was also dependant on the presence of magnesium ions, and the optimum magnesium concentration was shown to be 9 mM (in contrast to the reovirus optimum of 13 mM) in fig. 3(b). The optimum virus concentration was 750 $\mu\text{g/ml}$ [fig. 3(c)].

For use in comparative experiments (below), reovirus mRNA was obtained by in vitro transcription. The standard method of chymotrypsin digestion (Skehel and Joklik, 1969) was used to activate the virus particles, and the transcription mixture was the same as that used for rotavirus. A time course of transcription of reovirus is shown in fig. 4.

Fig.2 Reactivation of rotavirus transcription

400 μ g rotavirus was incubated in 0.5 ml transcription mix for 6h as previously described and a sample was counted for TLA insoluble radioactivity. The virus was then pelleted and resuspended in fresh transcription mix for three further reactions and the incorporated radioactivity was measured each time.

The yield of RNA from each reaction was as follows:

Reaction 1 = 220 μ g
 2 = 320 μ g
 3 = 75 μ g
 4 = 65 μ g

The total yield of RNA was 680 μ g.

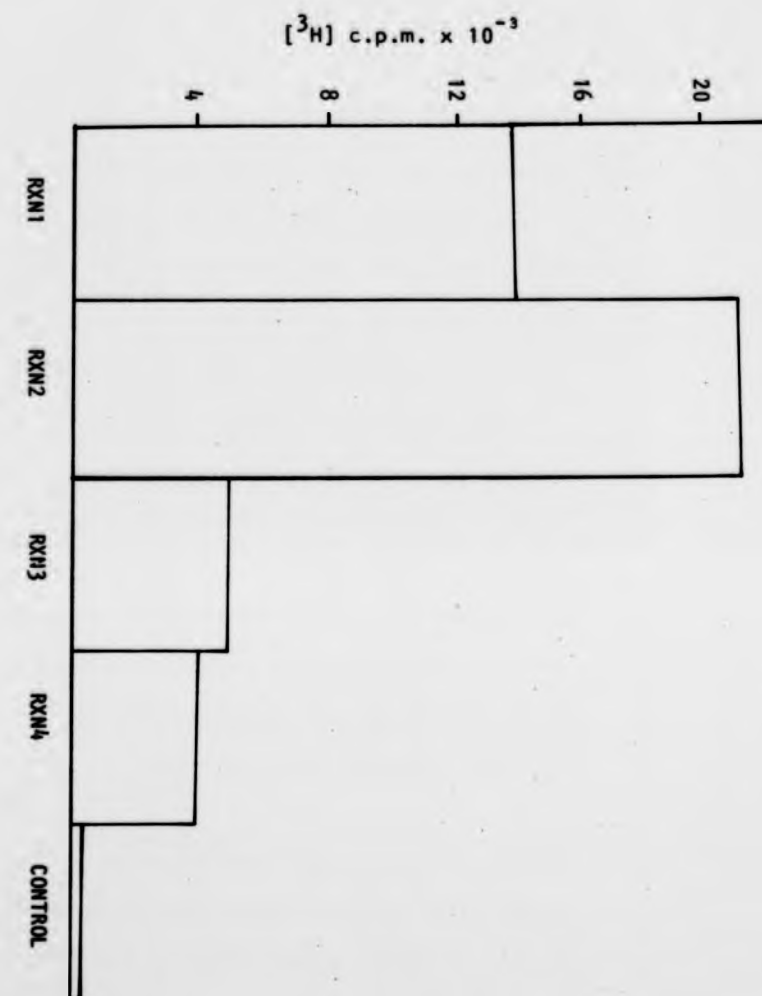


Fig.3 Optimisation of conditions for rotavirus in vitro transcription.

- a) Rotavirus was incubated in the full transcription mixture (■) or in mixture lacking GTP (◆), CTP (△) or ATP (○). The Control reaction contained no virus. (▲) Samples were taken at intervals and counted for TCA-insoluble radioactivity.
- b) Optimisation of Magnesium concentration for in vitro transcription of rotavirus. Rotavirus was incubated in the transcription reaction mixture containing the magnesium concentrations shown for 60 min and samples were then counted for TCA-insoluble radioactivity.
- c) Optimisation of virus concentration. The quantities of virus shown were incubated in 50 μ l of transcription reaction mix for 60 min, before samples were counted for TCA-insoluble reactivity.

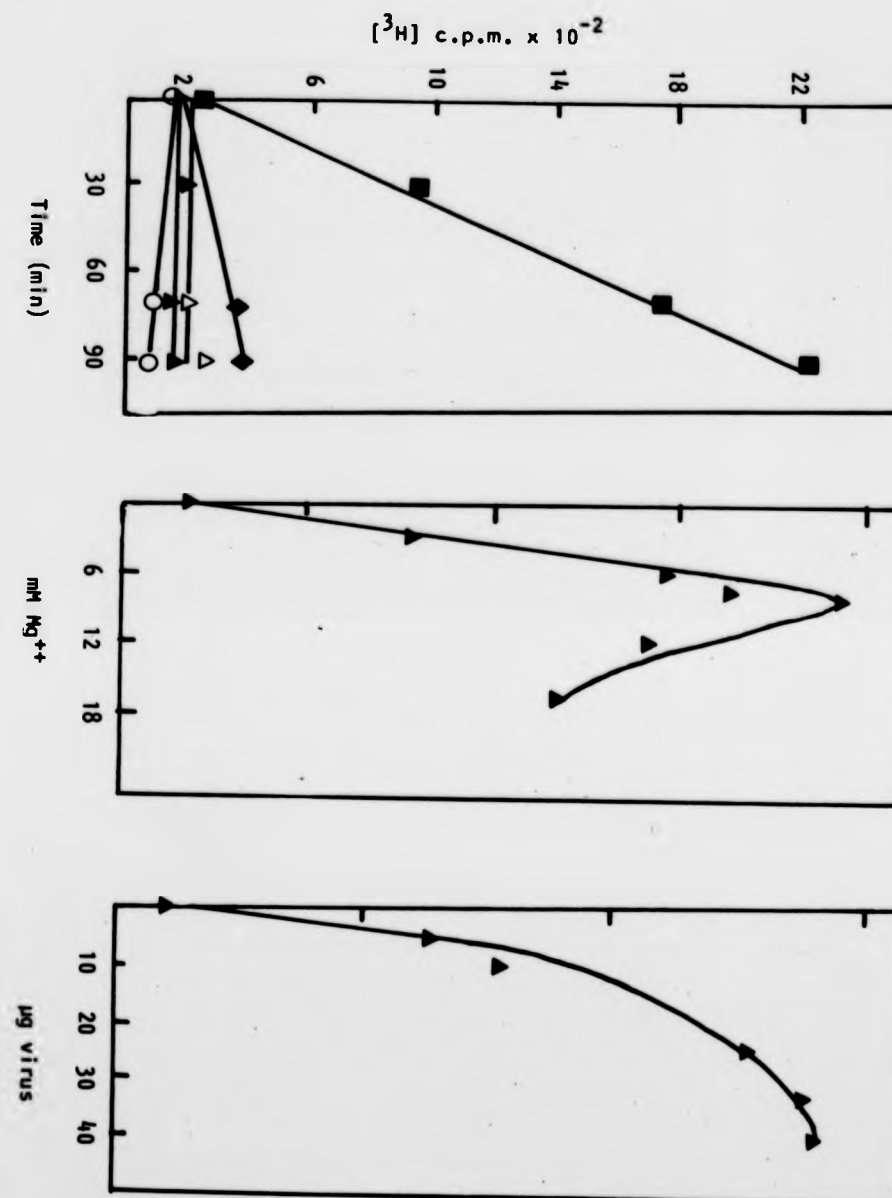
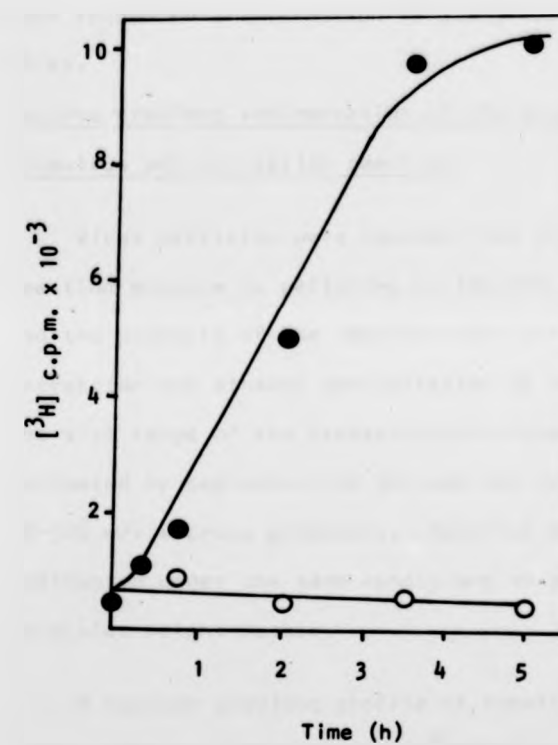


Fig.4 Time course of reovirus in vitro transcription.

Purified reovirus was digested with chymotrypsin and incubated in standard reaction conditions as described in materials and methods. Samples were taken at intervals and counted for TCA insoluble radioactivity.



3.1 (Cont'd.)

mRNA produced by in vitro transcription was purified, and the yield of mRNA was measured by OD_{280}/OD_{260} measurement. 1 mg of mRNA has OD_{260} of 5.60. 400 μ g of purified rotavirus transcribed 680 μ g of pure mRNA in four rounds of transcription (fig.2), i.e. 1.7 mg RNA per 1 mg virus.

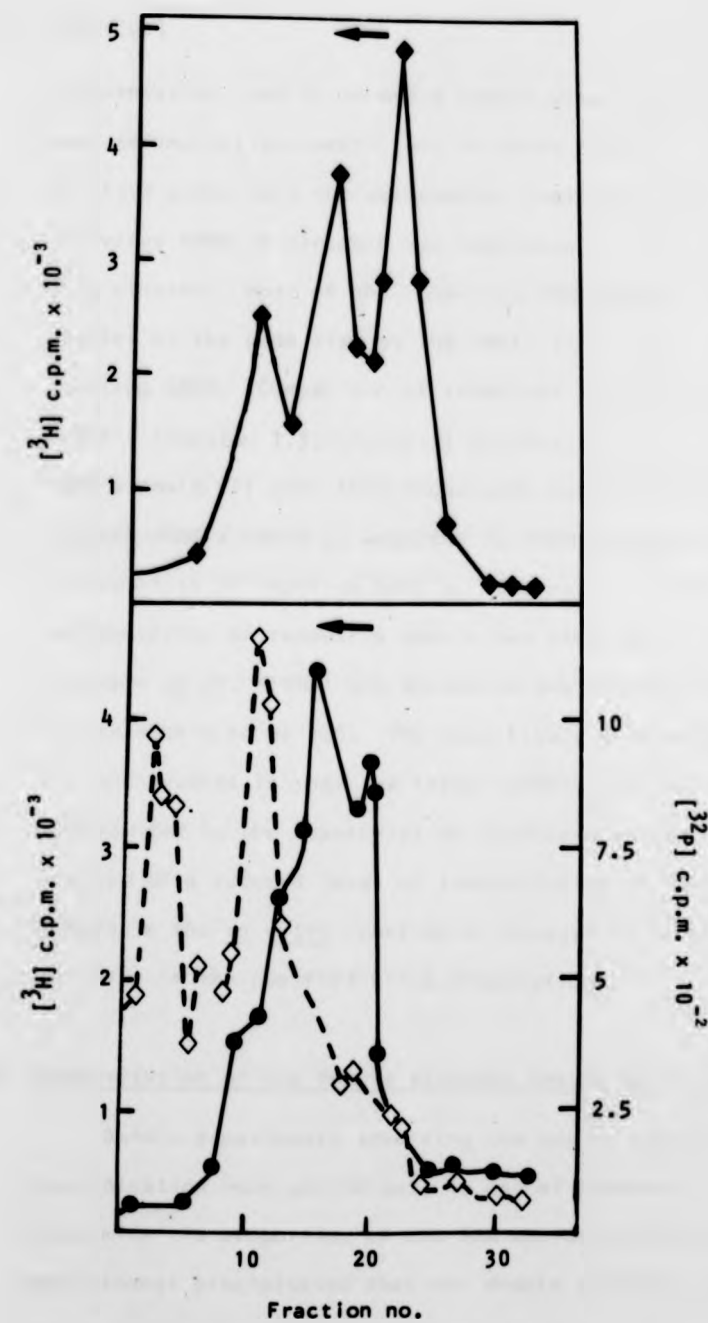
3.2 Sucrose gradient sedimentation of the products of the rotavirus polymerisation reaction.

Virus particles were removed from the transcription reaction mixture by pelleting at 100,000 xg for 60 min and the products of the reaction were purified by phenol extraction and ethanol precipitation of the supernatant. The size range of the transcription products was then estimated by sedimentation through non-denaturing linear 15-30% w/v sucrose gradients. Reovirus mRNA was sedimented under the same conditions to provide a molecular weight marker.

A sucrose gradient profile of reovirus mRNA transcribed in the presence of 32 P-GTP is shown in fig. 5(1), and the well characterised peaks of the s, m and ℓ size classes of reovirus mRNA are clearly seen. The peaks have mean sedimentation coefficients of 12S, 18S and 25S respectively (Skehel and Joklik, 1969). Rotavirus mRNA transcribed in the presence of 3 H-UTP was compared to reovirus mRNA by sucrose gradient

Fig.5

Sucrose-SDS density gradient fractionation of
a) ^3H -reovirus mRNA ($\text{---}\blacklozenge\text{---}$) fractionated for 18h
and b) ^{32}P reovirus mRNA ($\text{--}\diamond\text{--}$) and ^3H rotavirus
mRNA ($\text{---}\bullet\text{---}$) fractionated for 30h. The mRNA's from
in vitro transcription reactions were produced by
phenol extraction and ethanol precipitation and
layered onto 15-30% sucrose gradients in TLES
buffer. After centrifugation at 60,000g for the
times given at 20°C, the gradients were fraction-
ated into 0.3 ml samples. 50 μl aliquots of each
sample were counted for TCA precipitable c.p.m.
Direction of sedimentation is indicated by an
arrow at the top of the graphs.



3.2 (Cont'd.)

sedimentation, and it formed a single broad peak with a mean sedimentation coefficient of about 10S [fig. 5(ii)]. Discrete peaks were not detectable, indicating that rotavirus mRNA is probably not separable into discrete size classes. Most of the rotavirus mRNA appears to be smaller or the same size as the small size class of reovirus mRNA. Comparison of rotavirus and reovirus dsRNA's (section 1.3.1.4, fig.7) predicts that 7 of the rotavirus mRNA's would fit into this broad peak but that the four largest mRNA's would be expected to form a peak near the m size class of reovirus mRNA's. Sucrose gradient sedimentation of rotavirus mRNA's has since been done by Mason et al. (1980) who estimated the average size of the mRNA's to be 12S. The most likely explanation for our results is that the larger mRNA's are not well represented in the transcription products, either because of a reduced level of transcription of larger mRNA's in the in vitro reaction or because of ribonuclease activity in the purified virus preparations.

3.3 Demonstration of the double stranded nature of virion RNA

Before experiments involving the use of dsRNA for hybridisation were carried out, it was of interest to determine the proportion of the RNA extracted from virus and ethanol precipitated that was double stranded, and to determine if it was fully double stranded without ss

3.3 (Cont'd.)

tails. This experiment also tested whether or not denaturation of dsRNA in 90% DMSO separated the two strands and determined if our transcription product was single stranded RNA.

S1 nuclease is highly specific for single stranded RNA molecules or single stranded regions of RNA, and is frequently used to remove any ss tails on dsRNA molecules. dsRNA was 3' end labelled with ^{32}pCp and T_4RNA ligase as described in chapter 2. Free ^{32}pCp was separated from dsRNA by gel filtration on Sephadex G50. mRNA labelled in the presence of $^3\text{H-UTP}$, end labelled dsRNA and dsRNA which had been denatured in 90% DMSO for 20 min at 45°C and then ethanol precipitated were all incubated in the presence of 1,000 units/ml S1 nuclease. Samples were taken at 0, 15, 30 and 60 min and the TCA precipitable radioactivity in each sample was determined (table 1). Evidence of nuclease digestion was provided by the radioactivity in the RNA becoming TCA soluble. None of the TCA precipitable cpm in mRNA incubated without S1 nuclease were lost, while 96% of those in mRNA incubated with S1 nuclease were lost. All the TCA precipitable cpm in dsRNA were recovered after 60 min in S1 nuclease while only 3.3% remained in denatured dsRNA treated the same way. This experiment shows 1) that all the RNA extracted from virus particles is double stranded, 2) that there are no ss tails at the 3' end of the RNA's, 3) confirms that the denaturation conditions used are highly effective at separating the two strands of dsRNA and 4) it proves

Table 1 Effect of S1 nuclease on our transcription product.

Sample	Description	0'	c.p.m. ⁺ 15'	30'	60'	% loss
1	Control mRNA (No S1)	38,660	39,702	44,189	41,110	0
2	³ H mRNA	23,433	3,109	1,956	945	96
3	³² P dsRNA	99,554	88,157	90,628	110,080	0
4	³² P dsRNA denatured in 90% DMSO	168,093	16,769	9,681	5,567	96.7

⁺The samples indicated were incubated in no nuclease (no 1) or 1,000 units/ml S1 nuclease (samples 2, 3 and 4). Samples were taken at 15 min intervals and counted for TCA precipitable radioactivity.

3.3 (Cont'd.)

that the product of in vitro transcription is single stranded RNA.

3.4 Hybridisation of the polymerase product to virion RNA

This experiment was designed to give more detailed information on which RNA species are represented in the transcription products. Rotavirus mRNA transcribed in vitro was 3' end labelled and the end labelled RNA was separated from free ^{32}pCp by gel filtration. The end labelled mRNA was then hybridised to unlabelled genomic denatured dsRNA by the method of Ito and Joklik (1972). The mRNA was present in a 10-fold mass excess to maximise hybridisation of the radiolabelled product to each of the genome dsRNA segments. Samples were taken at timed periods and half of each were digested with S1 nuclease to remove single stranded RNA's. The radioactivity in both the digested and undigested samples was compared to determine the proportion of end labelled mRNA that had hybridised to dsRNA.

The end product of the hybridisation reaction was compared to dsRNA on polyacrylamide gels (PAGE) and the results are shown in fig.6. The rotavirus transcription products hybridised to virion dsRNA and the products comigrated with 3' end labelled dsRNA on PAGE. Analysis of the hybridisation products shows that the transcripts

Fig.6 Hybridisation of the rotavirus in vitro transcripts to rotavirus dsRNA.

Autoradiogram of SDS-PAGE of

- A) purified rotavirus ^{32}P labelled dsRNA and
- B) products of hybridisation of ^{32}P labelled transcripts to unlabelled denatured dsRNA.

3' end labelled mRNA was mixed with cold dsRNA for hybridisation as described in materials and methods. The products were ethanol precipitated, redissolved in 2x SSC buffer and treated with 0.5 $\mu\text{g}/\text{ml}$ pancreatic RNAase. The RNAase resistant material was then compared to ^{32}P labelled dsRNA by PAGE.

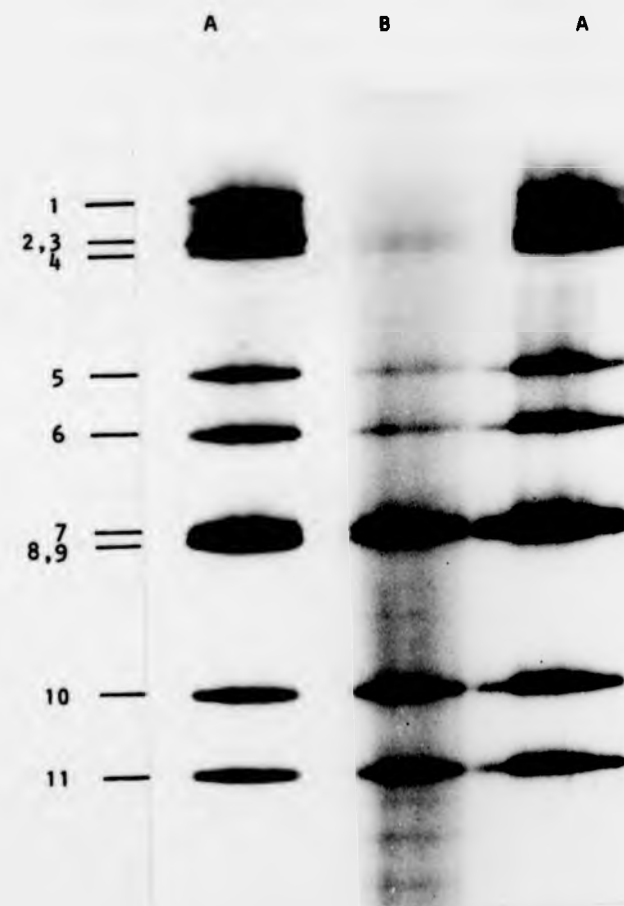
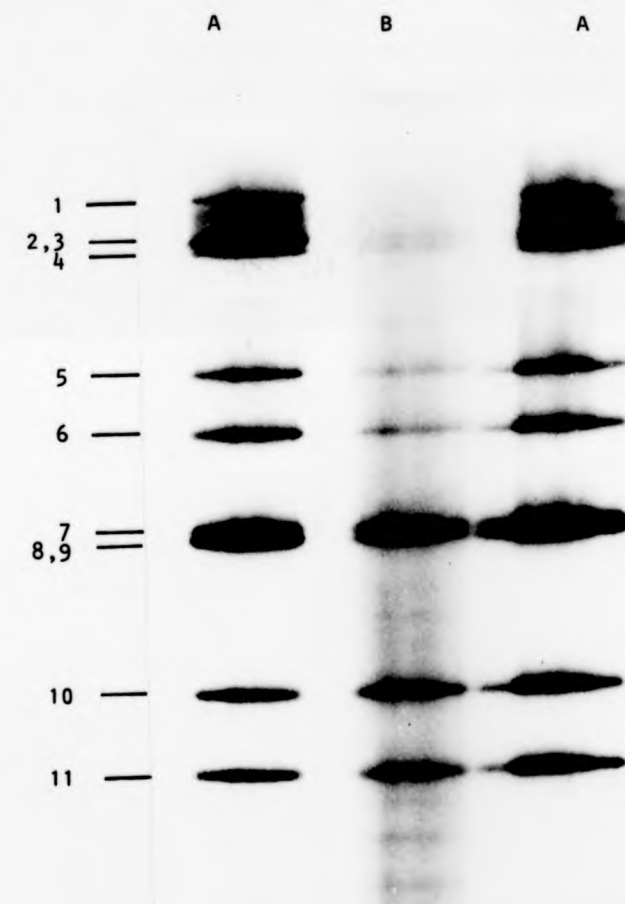


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3' end labelled mRNA was mixed with cold dsRNA for hybridisation as described in materials and methods. The products were ethanol precipitated, redissolved in 2x SSC buffer and treated with 0.5 $\mu\text{g/ml}$ pancreatic RNAase. The RNAase resistant material was then compared to ^{32}P labelled dsRNA by PAGE.



3.4 (Cont'd.)

of the larger RNA segments are under-represented. The experiment was also done with mRNA labelled with ^{32}P GTP in the transcription mix and the same results were obtained. This suggested that transcription of higher molecular weight RNA's was less efficient than that of lower molecular weight RNA's rather than the result of nucleases, as RNA fragments resulting from nuclease action would still hybridise to denatured dsRNA and some would be expected to form products that comigrate with dsRNA on PAGE. This result at least partially accounts for the small overall size of the mRNA product. In contrast, reovirus transcription synthesises equal mass amounts of RNA from each segment (Zweerink and Joklik, 1970), and transcription of reovirus under the same conditions as used for rotavirus produced mRNA of all three size classes in the expected proportions [see fig. 5(1)].

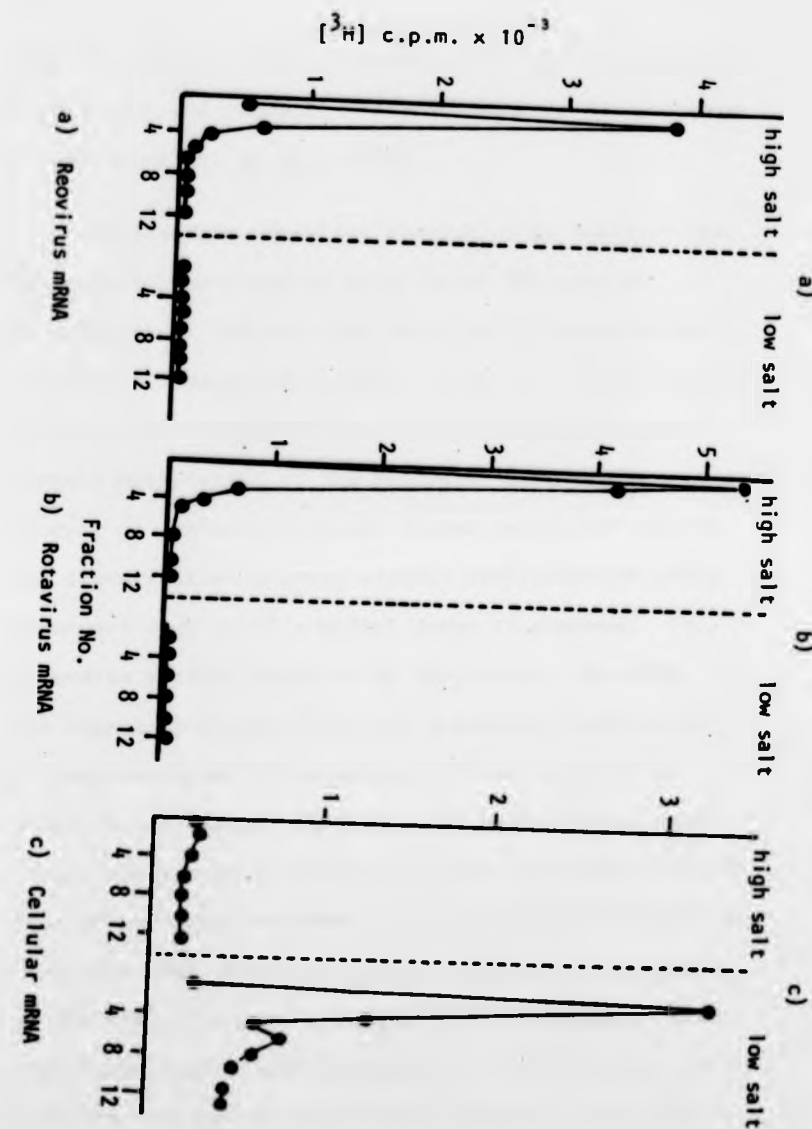
3.5 Analysis of posttranscriptional modifications of the in vitro RNA product.

3.5.1 Polyadenylation. The RNA transcribed in vitro by other members of the reovirus family is unusual in that it does not contain 3' terminal poly A (Stoltzfus et al., 1973; Bishop, 1977). We analysed the rotavirus RNA transcribed in the presence of ^3H -UTP by affinity chromatography on oligo dT cellulose to determine whether it contained a sequence of poly A. The results are shown in figure 7. Under conditions where 96% of the ^{32}P cellular mRNA (isolated from the cytoplasm of uninfected BSC-1 cells labelled with ^{32}P for 10h) bound to oligo dT cellulose, less than 1% of the rotavirus transcription product or reovirus mRNA bound to the column. Thus, rotavirus mRNA appears to resemble reovirus mRNA in that it does not contain 3' terminal poly A. The presence of a very short stretch of oligo adenylates has not been ruled out by this experiment—a further test of binding the mRNA to poly U filters (which bind much shorter stretches of poly A) would eliminate this possibility.

3.5.2 Methylation. The RNA polymerase activity of CPV is greatly stimulated by the inclusion of the methyl donor, S-adenosyl methionine in the reaction mixture (Furuichi, 1974). The methyl group of SAM is incorporated into a 5' terminal cap structure, similar to that found on most eucaryotic

Fig.7

Oligo dT cellulose chromatography of *in vitro* transcribed mRNA. ^3H rotavirus mRNA (centre panel) was compared to ^3H -reovirus mRNA (left panel) and ^{32}P -cellular mRNA (right panel). The purified mRNA in 0.15 ml 0.4M KCl, 50 mM Tris-HCl pH7.4 (high salt buffer) was washed through a column of oligo dT cellulose in high salt buffer. When all unbound radioactivity had passed through the column, mRNA which had bound to the oligo dT cellulose was eluted with 50 mM Tris-HCl pH7.4 (low salt buffer). Aliquots of each fraction collected were counted for TCA-precipitable radioactivity.



3.5.2 (Cont'd.)

mRNA's. Reovirus RNA polymerase activity is independent of SAM, but the products are methylated in the presence of SAM (Furuichi *et al.*, 1975).

The standard rotavirus transcription reaction was not greatly inhibited by omission of SAM, nor by replacement of SAM with the inhibitor of methylation. S-adenosyl homocysteine (SAH) (Fig. 8). These results indicate that rotavirus transcription can take place without methylation of the product. However, the product of transcription may become methylated during the transcription process without transcription being dependent upon it if a methyl donor is present. To determine whether methylation does occur, the mRNA was transcribed with ^3H -methyl S-adenosyl methionine as the radiolabelled precursor so that only methyl groups would become labelled. ^3H -methyl groups were indeed incorporated into newly transcribed mRNA (fig.8b). This methyl labelled mRNA co-migrated with ^{32}P -labelled rotavirus mRNA (which was transcribed in the presence of ^{32}P GTP) on sucrose gradient sedimentation (fig.9). These experiments confirmed that the rotavirus particle contains the enzymes required to methylate the product of rotavirus in vitro transcription.

Fig.8

- a) Effect of inhibiting methylation on transcription activity.

Virus was incubated in a) the standard reaction mix (—■—), b) mix lacking the methyl donor SAM (—●—) or c) mix where SAM was replaced by the inhibitor of methylation SAH (—▲—) (see text) and incorporation of ^3H -UTP into acid insoluble material was measured. Control samples (—◆—) contained no virus.

- b) Incorporation of ^3H -SAM into products of in vitro transcription.

Virus was incubated in a reaction mixture in which ^3H -UTP was replaced by ^3H -SAM (—▲—), and samples were counted for TCA-insoluble radioactivity. Control samples (—△—) contained no virus.

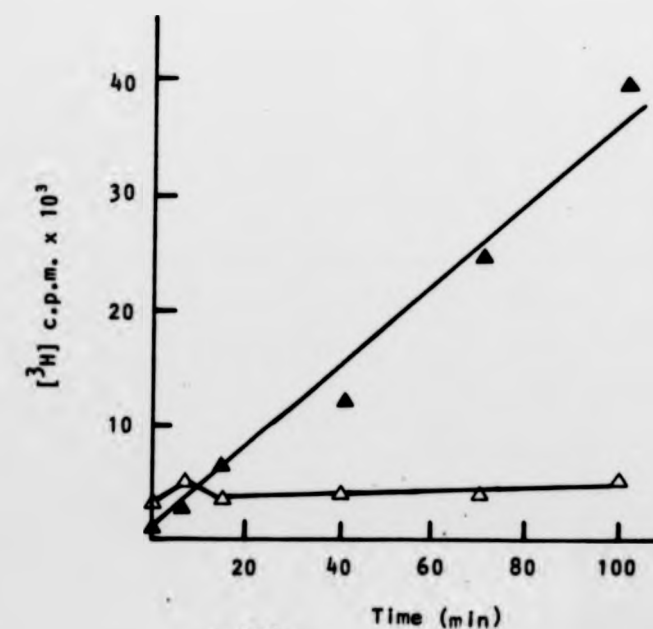
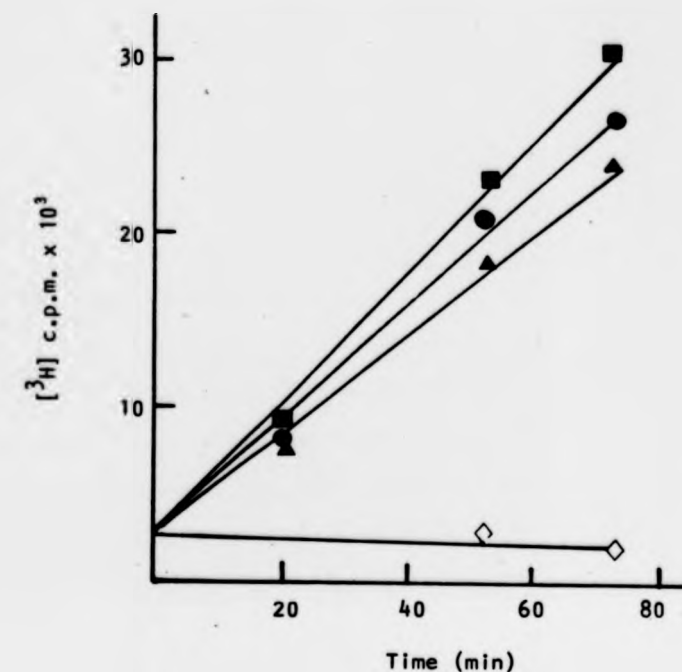
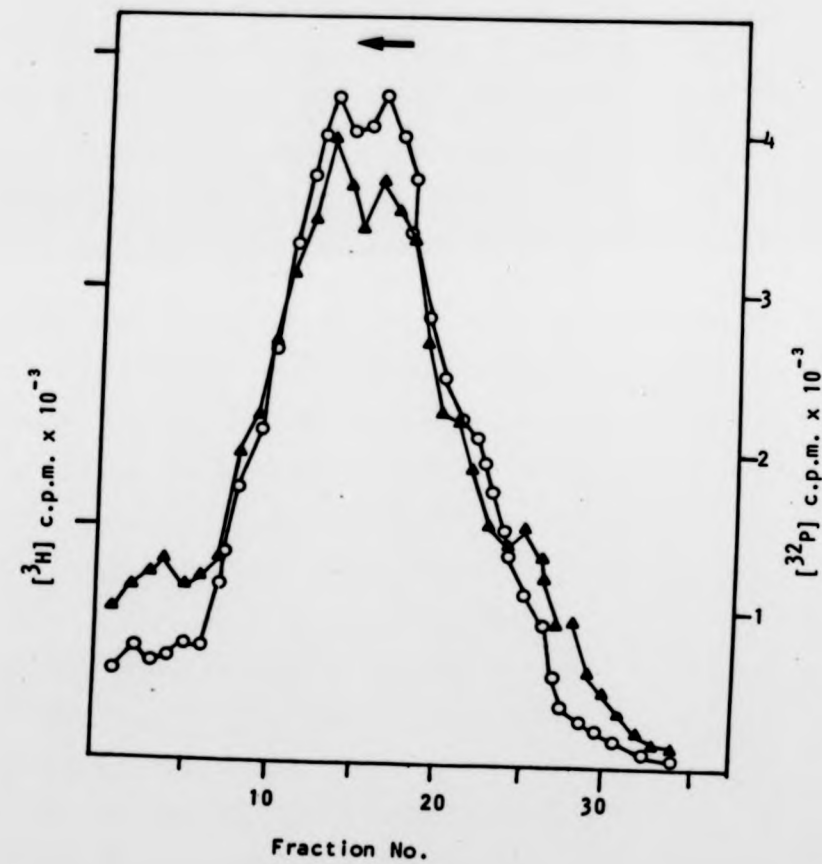


Fig.9

Sucrose-SDS density gradient fractionation
of a) ^{32}P rotavirus mRNA ($\rightarrow\Delta\rightarrow$) and
b) ^3H -methyl labelled rotavirus mRNA ($\rightarrow\bullet\rightarrow$).
The mRNA's were fractionated on a sucrose
gradient as described in fig.5.



3.5.3 Are the methyl groups present in an alkali resistant cap?

mRNA synthesised in the presence of ^3H -methyl S-adenosyl methionine was digested with alkali to determine whether the methyl groups were present in alkali digestible nucleotides or in an alkali resistant cap. After digestion in 0.3M KOH for 18h at 37°C, the products were fractionated by DEAE cellulose chromatography as described by Tener (1967) and compared with alkali digested mRNA transcribed in the presence of ^3H -UTP.

Partially digested 3' end labelled mRNA was used as a standard to give the elution positions of nucleotides of sizes ranging from mononucleotide to small oligonucleotides. The elution profile in fig.10 shows the positions of oligonucleotides with net negative charges from -2 to -6. When alkali digested ^3H -methyl labelled mRNA was subjected to the same conditions, most of the radioactive material was seen to have a net negative charge of between -5 and -6 from its position of elution from the column [fig. 11(i)]. Alkali digested mRNA labelled with ^3H -UTP was seen to have a net negative charge of -2. ^3H -methyl labelled reovirus mRNA, in which the methyl groups are known to be incorporated into a 5' cap, had the same elution profile as rotavirus mRNA labelled with ^3H -SAM [fig. 11(ii)]. These experiments therefore show

Fig.10

DEAE-cellulose chromatography of partially digested mRNA. A 'ladder' of marker oligonucleotides was prepared by partially digesting ^{32}pCp 3' end labelled mRNA with 50 mM carbonate/bicarbonate buffer pH9.0 at 90°C for 10 min. The digests were neutralized, diluted in 5 mM Tris-HCl pH7.8 and applied to a 5 ml DEAE cellulose column. A 50 ml linear gradient of 20 mM to 0.3 M NaCl in 7 M urea, 5 mM Tris-HCl pH7.8 was applied to elute the RNA's. The radioactivity in samples of each fraction was counted.

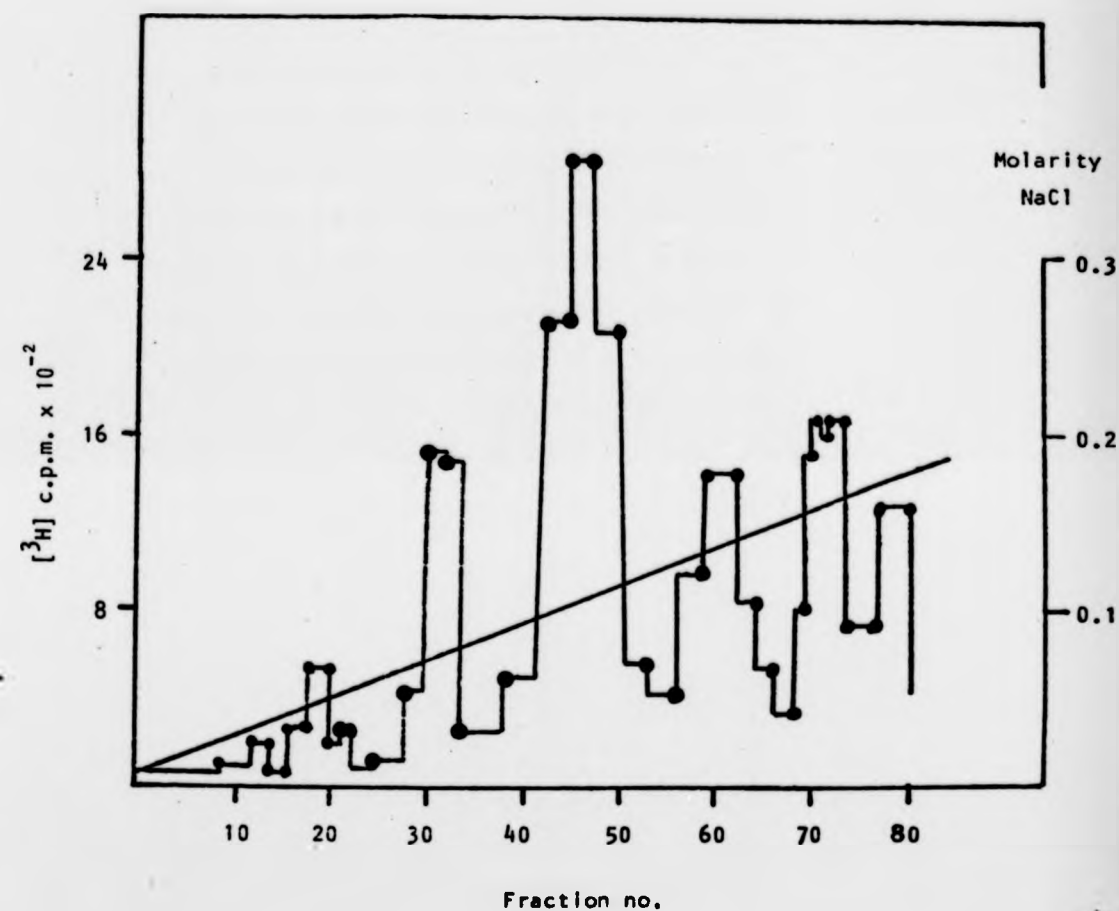
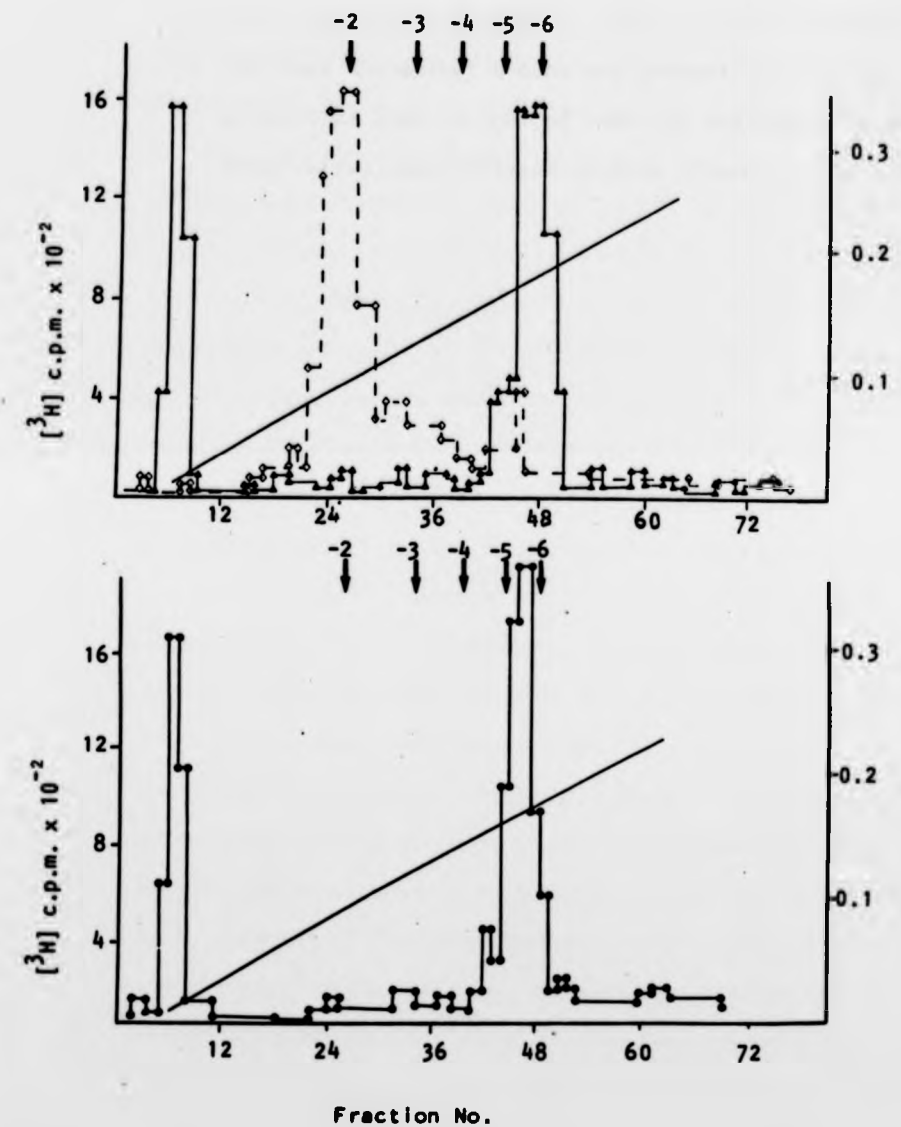


Fig.11

DEAE cellulose chromatography of alkaline digests of a) ^3H -UTP labelled rotavirus mRNA (---◇---) and ^3H -methyl labelled rotavirus mRNA (---▲---) and b) ^3H -methyl labelled reovirus mRNA (---●---). The mRNA's transcribed in vitro were digested with 0.3M KOH at 37°C for 18h. The digests were then neutralized, diluted in 5 mM Tris-HCl pH7.8 and fractionated on a 5 ml DEAE cellulose column as described in fig.10.



3.5.3 (Cont'd.)

that (1) rotavirus mRNA is capped and methylated and
(2) that the methyl groups are present in a 5' cap
of similar size to that of reovirus and indeed to all
other virus caps analysed to date (Shatkin, 1976).

CHAPTER 3

DISCUSSION

The aim of this part of the thesis was to characterise the RNA product of the calf rotavirus virion associated RNA polymerase. Structural characterisation of the RNA product is described in this chapter and the theme is extended in chapter 4 by determining its ability to function as mRNA in an in vitro translation system, as the primary motive for transcribing mRNA in vitro was to characterise the primary gene products by in vitro translation as a basis for studies on the processing of rotavirus polypeptides.

Conditions for efficient in vitro transcription of rotavirus were optimised. The well characterised reovirus polymerase was used for direct comparison of the transcription and properties of reovirus mRNA and rotavirus mRNA. The transcription reaction mix that we used was adapted directly from the one known to give optimal transcription of reovirus mRNA (Skehel and Joklik, 1969) and the only modifications were the magnesium concentration and the method of activation. Reovirus is activated by chymotrypsin which has to be removed prior to transcription but, because Cohen (1977) reported that the rotavirus polymerase was activated by EDTA which is a constituent of the transcription mix, we were able to cut out a separate activation step prior to rotavirus transcription. The reaction contains a bentonite-like reagent (Macaloid) which also activates the polymerase. Both compounds activate

transcription by chelation of calcium which loosens and removes the proteins of the virus outer shell (Cohen et al., 1979). The interesting feature of the transcription reaction itself was its great efficiency - the weight of products generated was greater than the mass of the input virus. Reovirus can transcribe mRNA with equal efficiency (Skehel and Joklik, 1969), which is part of the reason why reovirus mRNA's have been extensively used for the study of structure and function of eucaryotic mRNA's. The fact that the RNA product can be separated from the virion by centrifugation of the particles implies that the product is released from the particles during transcription. The core particle dsRNA is obviously conserved during the process and does not permanently combine with the new RNA product as the polymerase can be reactivated after the reaction by centrifugation of the virus and resuspending it in fresh transcription mix.

Proof that the products of the transcription reaction were indeed mRNA came from a number of experiments. Use of S1 nuclease proved that the product was single-stranded RNA. Sucrose gradient density sedimentation showed it to be of a size compatible with that expected if the dsRNA segments transcribed full length RNA copies. However, there was no peak compatible with the expected size of the four largest RNA segments so these are either under-represented or missing from our transcription product. Further investigation

using hybridisation showed that much less of these larger mRNA's hybridised to genomic RNA than the smaller mRNA's, revealing a deficiency in transcription from the larger genomic RNA species, in contrast to reovirus transcription, where all RNA's are transcribed at the same rate.

Bernstein and Hruska (1981) reached the same conclusion about rotavirus transcription - their quantitative estimates of each transcript after electrophoretic separation revealed that there was relatively less of transcripts 1, 2, 3 and 5 compared to other mRNA species. A possible remedy was reported by Mason et al. (1980) who found that when transcription times were reduced to 2h, in vitro translation of the transcripts produced more of the larger proteins.

These experiments showed that rotavirus transcribes eleven separate segments of single stranded RNA, each hybridising to one double stranded RNA segment. In vitro translation in chapter 4 was final proof that the transcripts act as mRNA.

A large section of this chapter was devoted to investigating the important modifications common on mRNA's - i.e. polyadenylation, methylation and 5' capping. Reovirus mRNA is one of a small group of functional mRNA's which completely lack a 3' poly A tail (Stoltzfus et al., 1973) and, while a more detailed analysis of the 3' terminal structure of rotavirus mRNA is needed before we can conclude that there

is absolutely no 3' poly A tract, it seems likely that rotavirus mRNA also belongs to this group as it does not bind to oligo dT cellulose. This observation has been confirmed by Bernstein and Hruska (1981). 3' poly A on mRNA has not been shown to have a function in recognition or in efficiency of translation but is reputed to be very important in protecting the stability of mRNA in vivo (Revel and Groner, 1978). However, reovirus mRNA seems to be as stable as any other mRNA species when microinjected into *Xenopus* oocytes (McCrae and Woodland, 1981). Thus reovirus mRNA (and presumably rotavirus mRNA) must have an alternative mechanism for ensuring stability of the mRNA species. However, both reovirus and CPV mRNA are methylated and have 5' caps (Bishop, 1977). We demonstrated incorporation of ^3H -S-adenosyl methionine into rotavirus mRNA during transcription which showed that it could be methylated in vitro and therefore that the purified virion carries an RNA methylase activity as well as the RNA polymerase activity initially described by Cohen (1977). More detailed analysis of the methyl labelled RNA by alkali digestion and anion exchange chromatography on DEAE cellulose revealed that the methyl groups on the RNA are present in the alkali resistant cap common to most eucaryotic mRNA's examined to date (Muthukrishnan et al., 1975). Methylation was not required for the transcription of rotavirus mRNA, making it likely that the capping mechanism will be similar to that seen with reovirus (Shatkin, 1974) and distinct from that

observed with the insect dsRNA containing virus CPV (Furuichi, 1978; see section 1.3.3.1.4). The presence of a 5' terminal cap structure implies the presence of another enzyme activity, namely guanyl transferase, which catalyses the 5' to 5' linkage of guanylic acid to the 5' end of the mRNA chain (Furuichi et al., 1979). Detailed sequencing of the cap structure is required to confirm this. The alkali resistant cap had the same net negative charge as the reovirus cap, so it probably has a type 1 cap structure as described by Shatkin (1976). Internal methylation often seen on eucaryotic cellular mRNA's but not found on reovirus or CPV mRNA was not detected in our analysis of ^3H -methyl labelled mRNA as no ^3H cpm comigrated with digests of RNA that were not alkali resistant.

The 5' cap of mRNA has an important role in both mRNA stability and in protein synthesis (Bannerjee, 1980). Capped mRNA's have been shown to be much more efficient than uncapped mRNA's in in vitro translation (Both et al., 1975; 1976) and the cap structure has been shown to facilitate efficient binding of reovirus mRNA's to 40S ribosomal subunits during initiation of protein synthesis (Both et al., 1976). It would be interesting to see if rotavirus has the same novel control mechanism as that of reovirus in that capped mRNA's are produced early in infection by parental virus and uncapped mRNA's are produced at later stages by the progeny virus (see Section 1.3.4.2).

CHAPTER 4

CHAPTER 4

Characterisation of rotavirus polypeptides.

Introduction.

There has been much recent work on the production, processing and topography of rotavirus polypeptides, as described in chapter 1.3.5, but at the commencement of this project the polypeptides were poorly characterised. Studies had been hampered by the difficulty of growing viruses in tissue culture, but this has now been successfully achieved with calf, pig and human rotaviruses (Mebus et al., 1971; Bridger and Woode, 1975; Thiel et al., 1977; Wyatt et al., 1980) helped by the discovery that trypsin enhanced rotavirus infectivity (Babluk et al., 1977). Early studies on rotavirus polypeptides analysed virus purified from faeces using PAGE and detected the polypeptides by staining (McNulty, 1979). Confusion was caused by variation in estimates of the number of virus proteins and of their molecular weights as well as by real variation in the extent of processing of the polypeptides and poor detection of minor polypeptides in stained gels. These problems made it necessary for us to characterise rotavirus polypeptides prior to the experiments described in chapters 5, 6 and 7.

Firstly, identification of the primary gene products by in vitro translation was used to identify the unprocessed polypeptides, as well as a test of the integrity of the mRNA that we transcribed in vitro (see chapter 3). Then rotavirus polypeptide synthesis in infected cells was studied by radiolabelling them with high specific activity ^{35}S -methionine, and structural

CHAPTER 4 (Cont'd.)

polypeptides of purified rotavirus particles were detected by 1) purifying virus grown in the presence of ^{35}S -methionine in tissue culture, 2) radiolabelling purified rotavirus in vitro with ^{125}I or 3) by Coomassie blue staining of unlabelled polypeptides. These were compared to the in vitro product. Finally, glycoproteins were identified with the aid of tunicamycin, an antibiotic which inhibits glycosylation of proteins (Schwarz et al., 1976). Since on PAGE of pulse labelled infected cells treated with tunicamycin the glycopolypeptide is absent and replaced by a smaller, non-glycosylated band, the glycoproteins and their precursors can be identified.

4.1 Identification of rotavirus polypeptides synthesised in infected cells.

Uninfected cells and cells infected with calf rotavirus at 10 p.f.u./cell were pulse-labelled with 50 μ Ci/ml 35 S-methionine for 30 min at 6h p.i. Proteins synthesised in both samples were compared by PAGE and autoradiography. Virus-induced polypeptides were defined as those seen in infected cells but not in uninfected cells, and these were presumed to be virus-coded as there is no evidence that rotaviruses switch on host protein synthesis. Up to 14 rotavirus polypeptides could be identified in infected cells (fig.1). The nomenclature used has been devised during work for this thesis as the published nomenclature systems were all unsuitable. Several recent reports have described polypeptides in infected cells, and these are all in general agreement with the pattern shown in fig.1., although most of them did not detect as many polypeptides (Thouless, 1979; Matsuno and Mukoyama, 1979; McCrae and Faulkner-Valle, 1981; Urquidí et al., 1981). Labelling the polypeptides VP (virus protein)1 to VP12 in order of decreasing molecular weight follows the scheme of McCrae and Faulkner-Valle (1981) for the same strain of calf rotavirus. The only deviation from this scheme is that VP3 and VP4 are reversed. This is because these two polypeptides are usually not resolved and the more intense and faster migrating polypeptide which forms a structural protein is called VP3 by all the other groups (mentioned above). These two polypeptides are also

Fig.1 Polypeptides of rotavirus infected cells.

PAGE of uninfected (Un) BSC-1 cells and cells infected (In) with calf rotavirus at 10 p.f.u./cell and labelled with [^{35}S]-methionine from 6-6.5h post infection. The polypeptides are labelled on the right hand side, and the nomenclature is explained in the text.

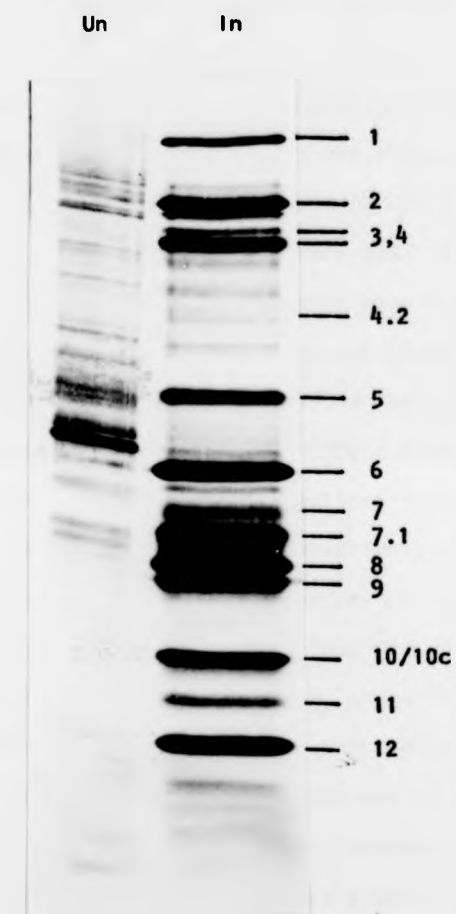
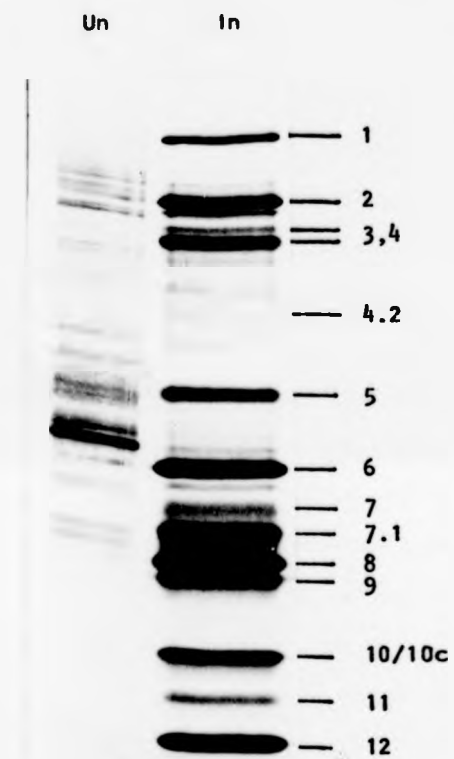


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4.1 (Cont'd.)

seen in vitro (fig.7). The virus proteins seen in fig.1 are consistently produced in infected cells, although minor differences can be seen on examination of other infected cell samples (see figs. 2, 3, 9 and chapter 5 figs. 5 and 7). The main sources of variation are 1) VP3 and VP4 are not always resolved, 2) VP11 is rarely seen 3) VP12 is often missing 4) VP10/VP10c are usually seen as two polypeptides with the faster migrating of the two being more intense and 5) in some cases, only one of VP7 or VP7.1 are detected. Both VP10 and VP10c and VP7 and VP7.1 are related proteins and in both cases McCrae and Faulkner-Valle (1981) showed by pulse-chase analysis the smaller polypeptide was derived from the larger. They were thus called VP10 and VP10c (c for chase) and VP7 and VP7c (our VP7 and VP7.1). The nomenclature VP7c was not used as there is also a smaller polypeptide related to VP7.1, namely VP7.2 (see chapter 6, fig.8). After pulse-labelling periods of 15 min, both VP10 and VP10c were always detectable (however, they are not resolved in fig.1), but VP7.1 was usually only found after longer (30-60 min) periods of labelling. This implies that the processing of VP10 is much faster than that of VP7, and/or that the synthesis of VP7 was faster.

Molecular weights of the polypeptides, estimated with the aid of reovirus protein as markers are listed in table 1.

Table 2 Molecular weights of reovirus proteins. The reovirus proteins that were used for comparison are listed on the right.

Protein species VP	Molecular weight	Reovirus protein	Molecular weight
1	125,000	$\lambda 3$	135,000
2	98,000		
3	82,000	$\mu 1$	80,000
4	86,000		
3*	76,000		
4*	74,000	$\mu 1c$ $\mu 2$	72,000 70,000
4.2	62,000		
5*			
5			
6	42,000	$\sigma 1$	42,000
7	38,000	$\sigma 2$	38,000
7.1	36,000	$\sigma 3$	34,000
7.2	33,000		
8	32,500		
9	32,000		
vpr7	31,000		
10	27,000		
10c	26,500		
11	24,500		
11c	24,000		
12	20,000		

4.1 (Cont'd.)

On shorter exposure of gels (see figs. 2 and 9) variation in intensity of the bands in infected cell extracts are easily seen. The most prominent virus band is VP6 followed closely by VP8 (and VP12 in fig.1), then VP7/7.1. VP2 and VP3 are always prominent while VP1, VP4, VP5, VP9 and VP10/10c are weak bands (although VP10/10c is variable in intensity) and VP4.2 and VP11 are barely if at all visible. This pattern is reproducible in cells infected with calf rotavirus - the main variation is in the quantity of VP10/10c and VP12 (VP12 is often absent from infected cells) and in the ratio of VP7 and VP7.1. A quantitative estimate of these observations (taken from a different gel) is seen in chapter 5, table 1. The results agree with the observed pattern but it must be noted that these values simply represent the amount of methionine incorporated into each polypeptide and, as each protein varies in methionine content, this does not represent the quantity of each protein being produced. However, as stained and methionine labelled virus bands have the same general pattern (see section 4.6) these observations may reflect real variation in the quantity of individual proteins.

4.2 Kinetics of virus protein synthesis in infected cells.

The kinetics of virus protein synthesis were estimated by pulse labelling infected cells for 15 min at hourly intervals. These cells were only infected with 5 p.f.u./cell,

4.2 (Cont'd.)

so host cell protein synthesis was not completely shut off but the background was low enough to clearly detect the virus proteins emerging (fig.2). Virus proteins were first detected 2h after infection, and there were no early and late stages of synthesis - once synthesis of any protein began, this protein was actively synthesised until late in infection. Some proteins were synthesised earlier than others, so some temporal control takes place. VP6 was the first to become visible and was easily seen after only 2h. VP8 and VP9 were just discernable after 3h and VP1, VP2, VP3 and VP7 by 4h after infection. The synthesis of VP10 and its chase product VP10c or VP12 were not noted until 5h after infection. The synthesis of VP4 and VP5 was difficult to determine as both comigrated with an uninfected cell polypeptide. Virus proteins were still being synthesised at 11h after infection (not shown in this fig.). The chase product of VP10 but not of VP7 was visible after a 15 min labelling period, so cleavage of VP10 appears to be faster than that of VP7.

Since the intensity of individual proteins differed (see also 4.1) and the times of appearance also varied, the relative amounts of each protein being synthesised was evidently under translational and/or transcriptional control, although differences in methionine content could account for some of this. Differential transcription in vitro has been reported

Fig.2

Time course of rotavirus polypeptide synthesis
in infected cells.

PAGE of uninfected (U) cells and cells labelled
at 1, 2, 3, 4, 5, 6, 7, 8.5 and 10h post infection.
Numbers on the right refer to rotavirus polypeptides.
(a) on the left refers to actin.

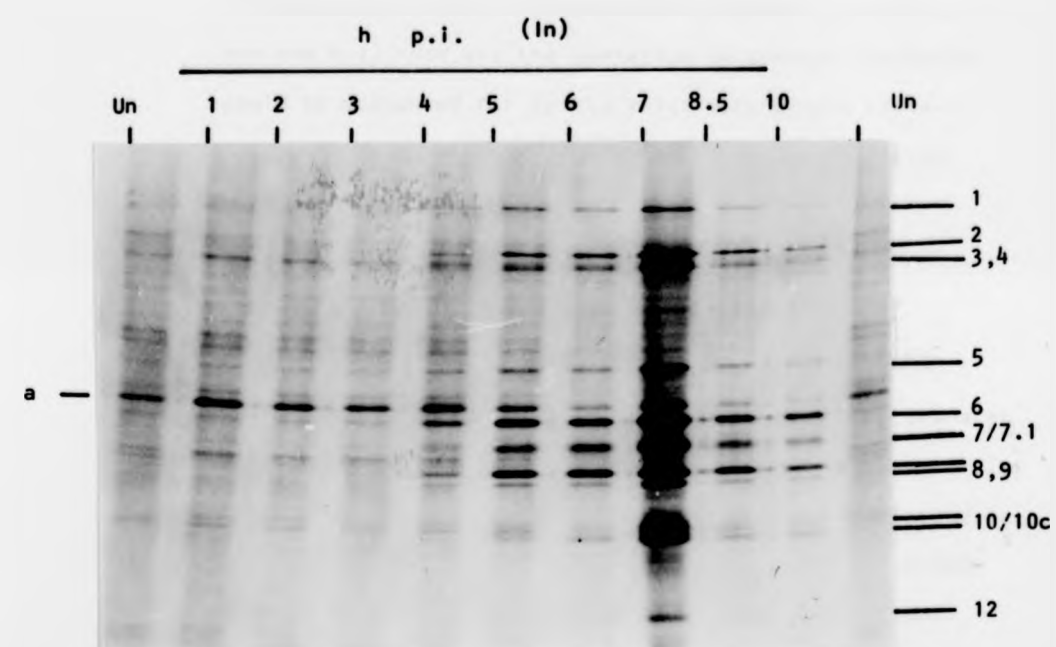
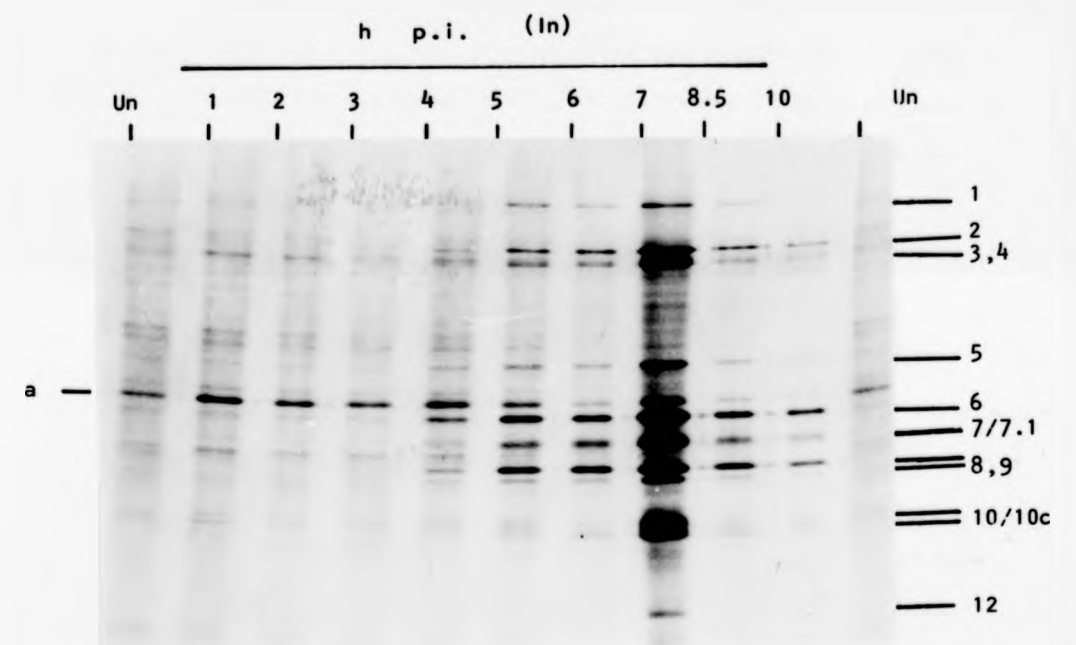


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Numbers on the right refer to rotavirus polypeptides.
(a) on the left refers to actin.



4.2 (Cont'd.)

by Bernstein and Hruska (1981) who found relatively less transcription of genome segments 1, 2, 3 and 5 (especially segment 1), so if the same situation occurs in vivo, this could account for differences in quantities of protein synthesised. From analysis of relative amounts of ³⁵S-methionine c.p.m. in each band (chapter 5, table 1, and see 4.1), not all the variation in protein synthesis could be accounted for by the relatively simple transcriptional control described so there is probably also some translational control.

4.3 Identification of rotavirus glycoproteins and their precursors by tunicamycin treatment of infected cells.

Tunicamycin inhibits glycosylation by blocking the transfer of carbohydrate chains, which have been assembled on carrier lipids, to the protein acceptor. The action is effected as tunicamycin is a lipophilic analogue of the normal oligosaccharide donor UDP-N-acetyl-glucosamine (Schwarz and Datema, 1980). In this experiment, the glycoproteins were identified by comparison of infected cells with those in which glycosylation was inhibited by 5 µg/ml tunicamycin throughout the infection and radiolabelling period.

Fig.3 shows that the rotavirus proteins VP7, VP7.1, VP10 and VP10c were absent from cells treated with tunicamycin and in the same cells there was a concomitant increase in VP12 and there was also a new protein migrating just below

Fig.3 Identification of rotavirus glycoproteins.

BSC-1 cells infected with 10 p.f.u./cell calf rotavirus (In) in the absence (-T) or presence (+T) of 5 µg/ml tunicamycin (which inhibits glycosylation) were labelled with ³⁵S-methionine at 6-7h p.i. Uninfected (Un) cells were maintained in the absence (-T) or presence (+T) of tunicamycin and radiolabelled at the same time.

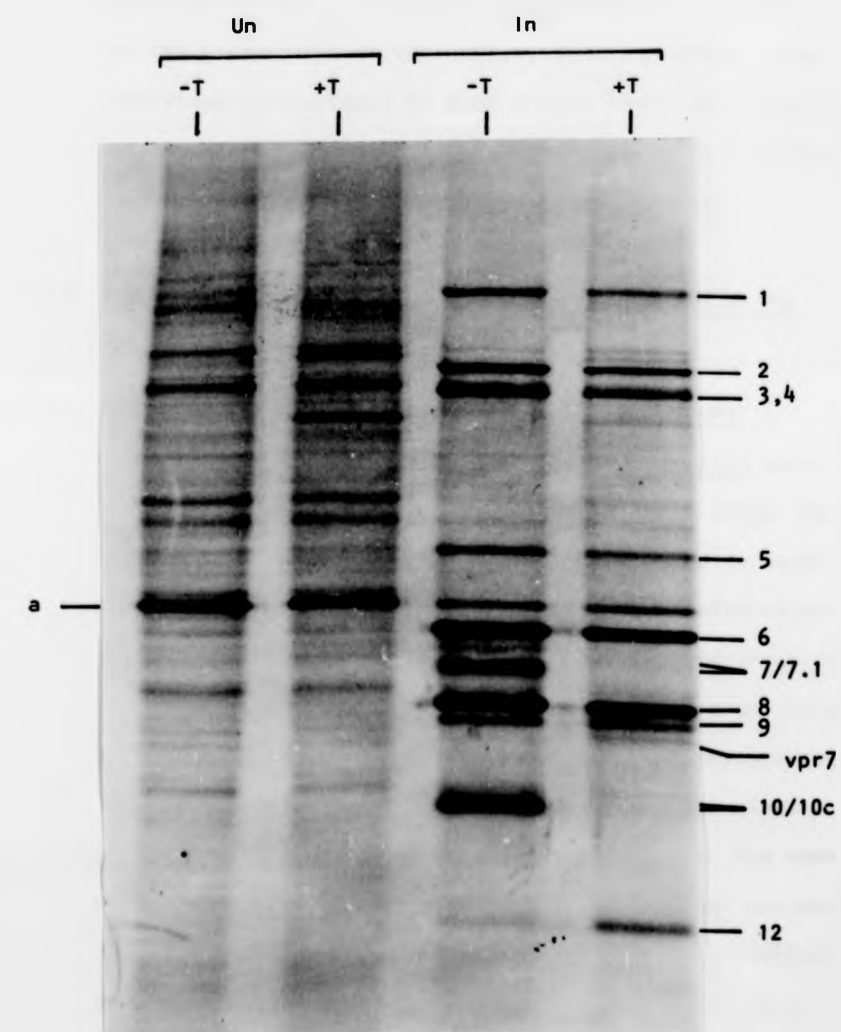
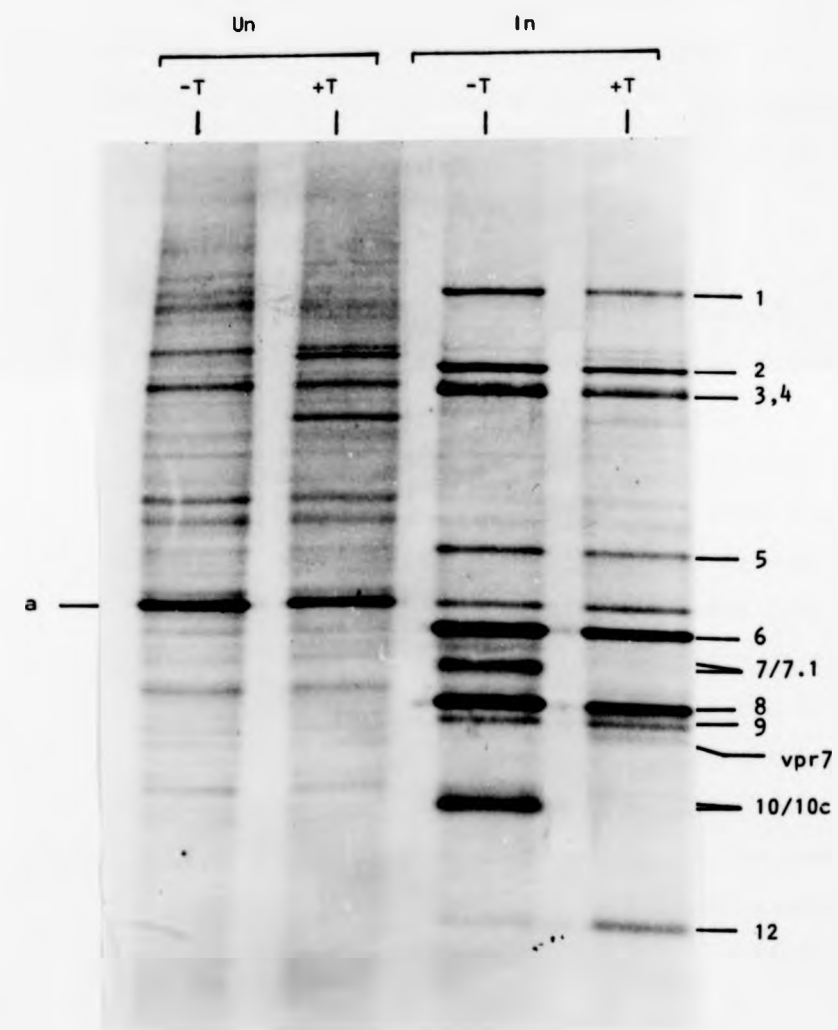


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4.3 (Cont'd.)

VP9 (labelled vpr7 by McCrae and Faulkner-Valle, 1981). vpr7 and VP12 were not seen in uninfected cells treated with tunicamycin, so they are identified as precursors of the glycoproteins VP7, VP7.1, VP10 and VP10c. The individual precursors of each glycoprotein are distinguished in chapter 6. These results confirm the work of McCrae and Faulkner-Valle (1981) and Arias et al. (1982).

4.4 Identification of primary gene products by in vitro translation of rotavirus RNA.

4.4.1 Optimisation of translation reaction conditions

The products of rotavirus transcription in vitro were translated in rabbit reticulocyte lysates to prove that these products were mRNA (see chapter 3), and in order to compare the rotavirus primary gene products with virus polypeptides synthesised in infected cells and those found in virus particles. The comparison would give an indication of the extent of processing of the primary gene products that takes place in vivo to produce functional virus particles. Reovirus mRNA was translated under the same conditions as a positive control. The reaction was monitored by incorporation of ³⁵S-methionine into TCA precipitable products, which was found to be 10-20 times higher in lysates with added virus mRNA than in control lysates (see figs. 4, 5 and 6). Our rotavirus mRNA was as effective as reovirus mRNA

4.4.1 (Cont'd.)

at stimulating translation (fig.4a). The initial rate of translation was dependent on the concentration of mRNA and on the translation mix. Increasing the mRNA concentration from 0-40 $\mu\text{g/ml}$ led to a greater initial rate of translation but there was no increase from 40-80 $\mu\text{g/ml}$ RNA (fig.5). When a freshly made and an older translation mixture (i.e. containing amino acids and an energy source which had been frozen at -20°C) were compared, the fresh mix allowed a higher initial rate of translation but the final level of incorporation did not vary (fig.4b), indicating that provided enough time for the reaction was allowed, it was safe to use frozen stocks of amino acids and energy source. Incorporation of ^{35}S -methionine was seen to occur at a linear rate for 20-30 min and then decline.

Conditions for the translation of rotavirus mRNA were optimised as shown in fig.6. Concentrations of KCl and Mg^{++} strongly influenced the incorporation of ^{35}S -methionine and were optimal at 125 mM and 2.25 mM respectively. These figures include the base levels of 35 mM KCl and 1.25 mM Mg^{++} already present in the lysate. As mentioned above, a concentration of 50 $\mu\text{g/ml}$ input RNA was found to be saturating - any further RNA did not increase the amount of polypeptide synthesised.

Fig. 4

- a) Incorporation of ^{35}S -methionine into acid insoluble products.

Rotavirus mRNA (\blacktriangle) and reovirus mRNA (\bullet) were incubated in the rabbit reticulocyte lysate as described in Materials and Methods. Control lysates (\circ) had no added mRNA. Samples were taken at intervals and counted for TCA-insoluble radioactivity.

- b) Rotavirus mRNA was incubated in lysate containing a fresh (\blacksquare) and frozen (\bullet) stock of amino acids and energy source. Control lysate (\circ) contained no mRNA.

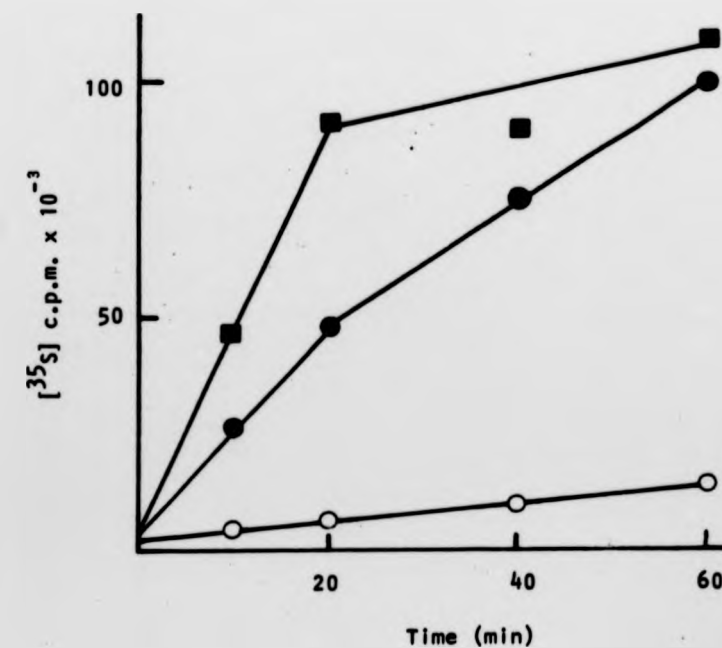
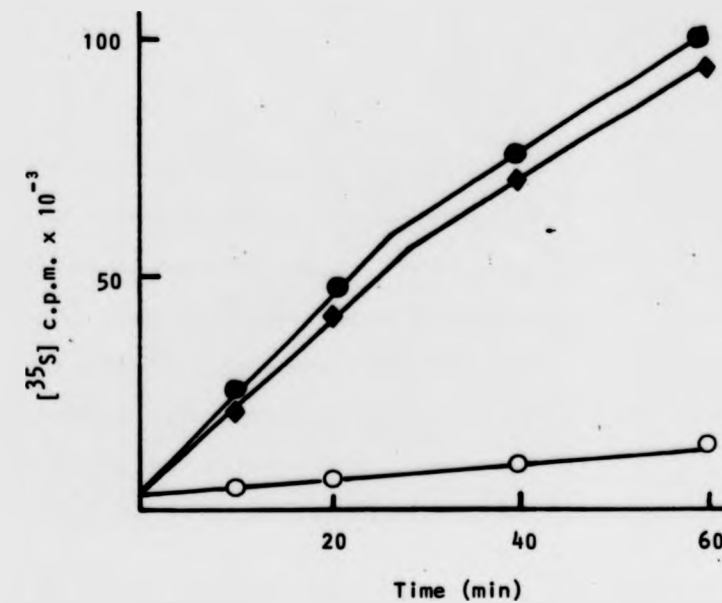


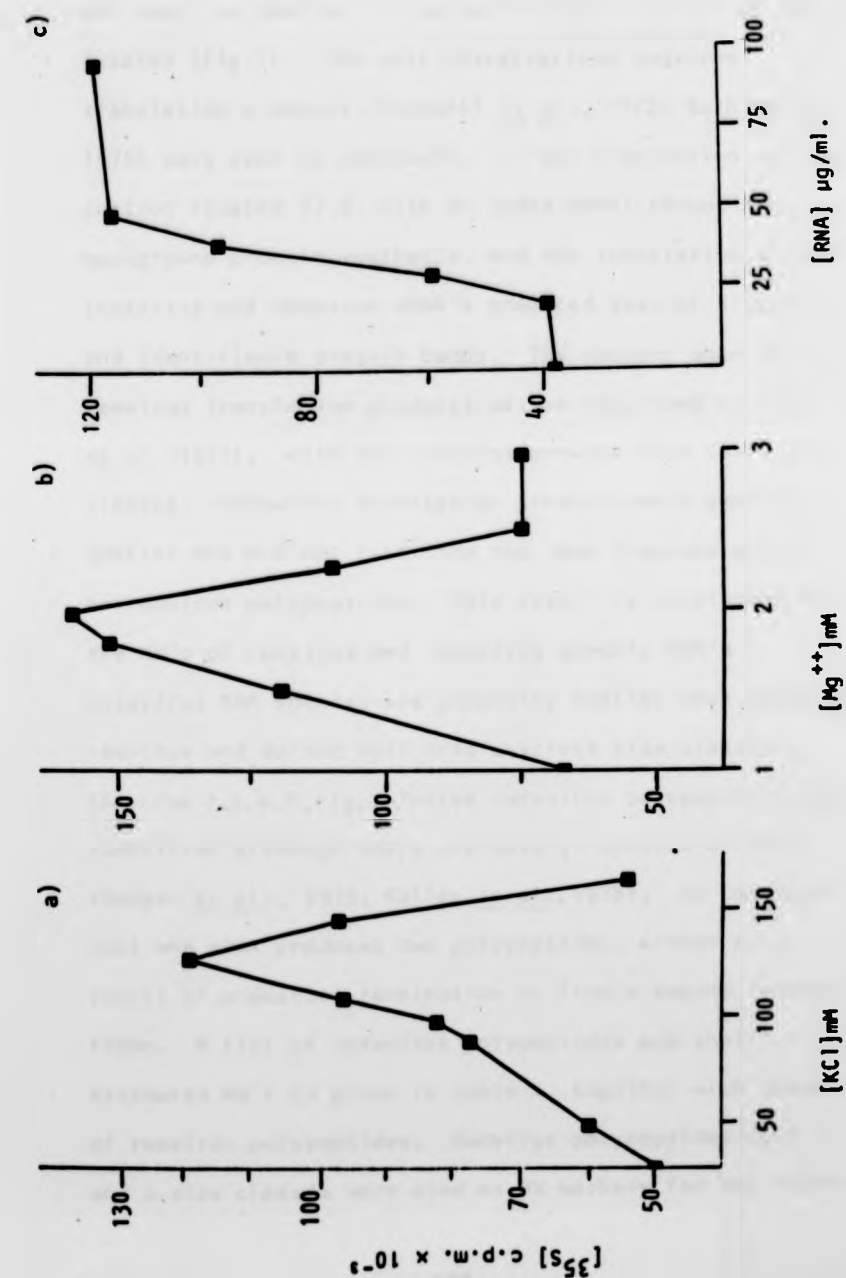
Fig.5

Time course of in vitro translation containing different concentrations of mRNA. Rabbit reticulocyte lysates were incubated with no (-○-), 20 µg/ml (-◇-), 30 µg/ml (-▲-), 40 µg/ml (-●-) and 80 µg/ml (-■-) mRNA. Samples of each were taken at intervals and counted for TCA-insoluble radioactivity.



Fig.6 Optimisation of the in vitro translation reaction.

- a) Rotavirus mRNA was incubated in lysate containing the concentrations of KCl indicated for 60 min, and samples were then counted for TCA-insoluble radioactivity.
- b) mRNA was incubated in lysate with the concentrations of Mg-acetate shown for 60 min before samples were counted.
- c) The quantities of RNA indicated were incubated in the lysate for 60 min, and samples were counted.



4.4.2 Identification of rotavirus primary gene products.

The products of in vitro translation of rotavirus mRNA and reovirus mRNA were compared by PAGE analysis of the lysates (fig.7). The well characterised reovirus translation products (McDowell et al., 1972; Both et al., 1975) were used as standards. In our translation system, control lysates (i.e. with no added mRNA) showed no background protein synthesis, and the translation of both rotavirus and reovirus mRNA's produced several discrete and identifiable protein bands. The pattern seen in reovirus translation products was as described by Both et al. (1975), with the proteins grouped into three size classes. Rotavirus translation products were generally smaller and did not fall into the same discrete groups as reovirus polypeptides. This result is consistent with the MW's of reovirus and rotavirus genomic RNA's - rotavirus RNA species are generally smaller than those of reovirus and do not fall into discrete size classes (Section 1.3.4.1, fig.7). Twelve rotavirus polypeptides were identified although there are only 11 species of RNA (Rodger et al., 1975; Kalica et al., 1978). We conclude that one mRNA produces two polypeptides, either as a result of premature termination or from a second reading frame. A list of rotavirus polypeptides and their estimated MW's is given in table 1, together with those of reovirus polypeptides. Reovirus polypeptides of λ and μ size classes were used as MW markers for the higher

Fig.7 Rotavirus polypeptides seen after in vitro translation.

The products of rotavirus in vitro translation (T) are compared to reovirus in vitro translation products (RT) and cells infected with calf rotavirus at 10 p.f.u./cell (In). The uninfected cell pattern is shown in Un and the products of the in vitro translation reaction without added mRNA is shown in CT. 6b is a longer exposure of the gel shown in 6a to show high molecular weight translation products (arrowed). X and Y are also arrowed. The virus proteins are labelled on the left hand side of 6a and 6b.

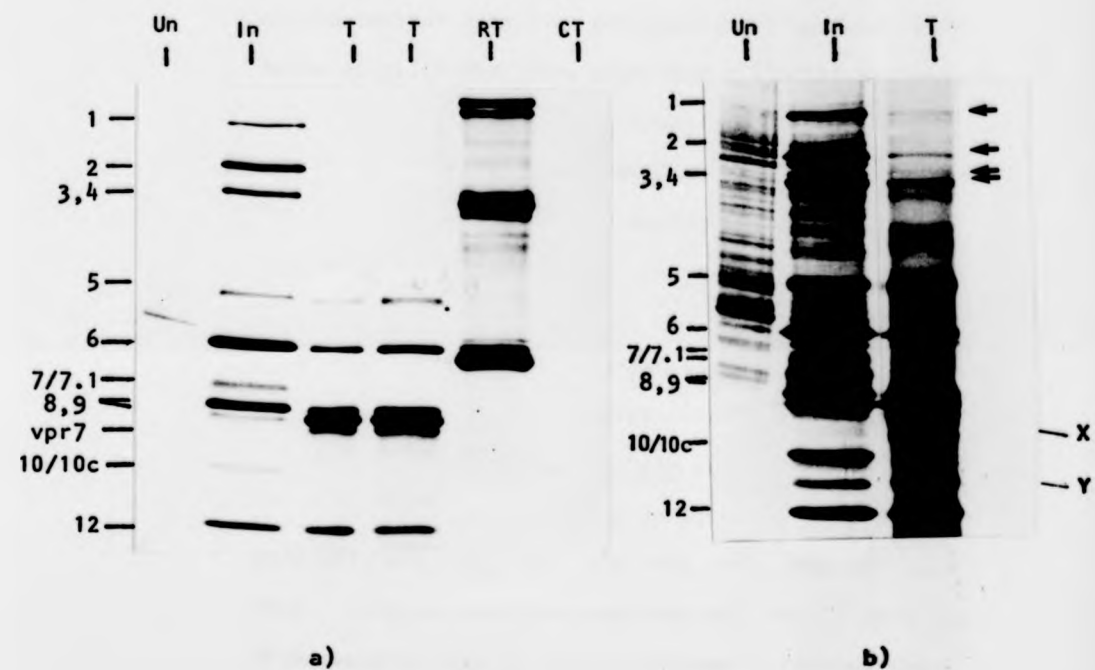
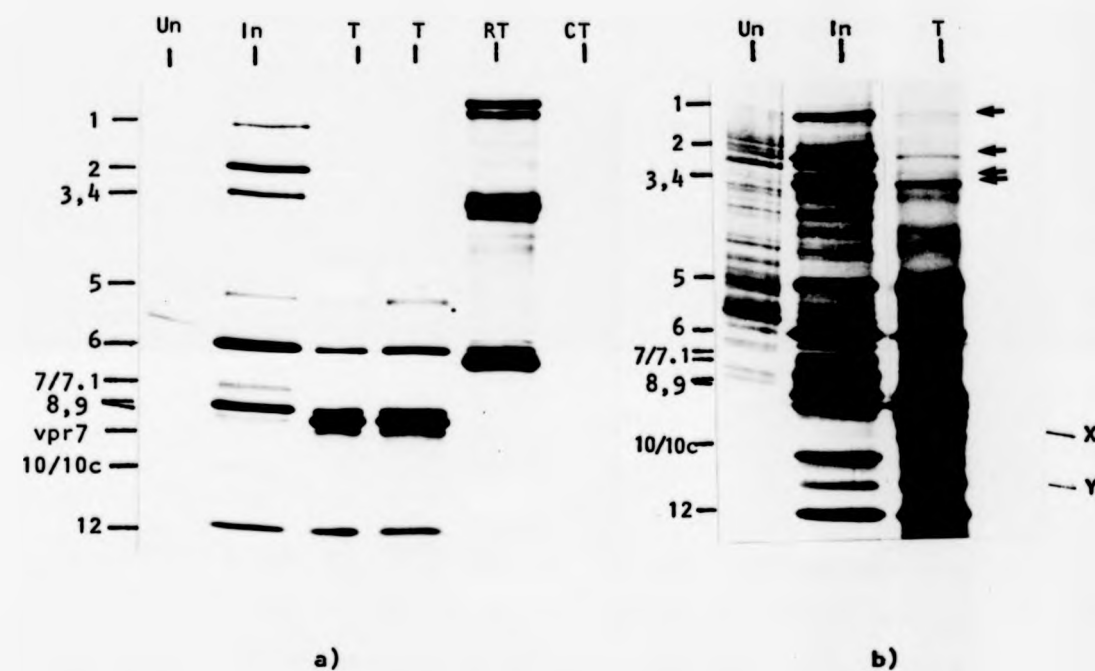


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4.4.2 (Cont'd.)

MW rotavirus polypeptides. However, there are discrepancies between reported MW's of reovirus σ proteins (Both et al., 1975) and the reported MW's of the smaller rotavirus polypeptides (Thouless, 1979). Mason et al. (1980) have also seen a similar pattern of polypeptides after in vitro translation of the simian rotavirus SA-11, but they found one extra band migrating ahead of VP3,4 which was an aggregate of a smaller polypeptide.

The rotavirus translation products were also compared to the proteins of infected cells. Ten of the twelve polypeptides seen after in vitro translation comigrated with rotavirus proteins seen in infected cells - these were VP1, VP2, VP3, VP4, VP5, VP6, VP8, VP9, VP11 and VP12. Infected cell polypeptides VP7, VP7.1, VP10 and VP10c were not seen in vitro, presumably because they are glycosylated, and two polypeptides (x, y) were seen in vitro but not in infected cells. One of these (x) is probably identical to vpr7 found in tunicamycin-treated infected cells. vpr7 and VP12 are precursors of the glycoproteins VP7, VP7.1, VP10 and VP10c (section 4.3) and are primary gene products. Polypeptide (y), which is only seen in vitro, is thus the extra primary gene product produced from one of the mRNA species.

4.4.2 (Cont'd.)

Because rabbit reticulocyte lysates are reported to allow some post-translational modification due to proteases present in the system (Shatkin, 1977), our mRNA was also also translated in a wheatgerm translation system as it is said to have little or no proteolytic activity (fig.8). The same proteins were synthesised in both systems, confirming that these were primary gene products. However the wheatgerm system is seen to be less efficient in translation of high molecular weight mRNA's.

4.5 Comparison of rotavirus polypeptides seen in vitro and in vivo by peptide mapping.

Single dimension peptide mapping was used to compare proteins from infected cells and in vitro translation in order to verify the authenticity of the translation products. The peptide mapping procedure requires that there is both an equal quantity and an equal radioactive intensity of the proteins to be compared, as a standard quantity of protease is used and this will not digest unequal quantities of protein to the same extent. In this case, it was impossible to use equal quantities of polypeptide as they are derived from different sources and differ in the specific activity of incorporated radiolabel. We therefore set up two separate digestion reactions for each protein - using 100 µg/ml and 10 µg/ml Staphylococcus aureus V8 protease - and the peptides from

Fig.8 Comparison of the products of in vitro translation of rotavirus mRNA in the wheatgerm and rabbit reticulocyte lysate systems. A sample of the wheatgerm system (W/G) and the rabbit reticulocyte lysate (RR) were taken after in vitro translation (T) and compared to infected cells (In). The rotavirus-induced polypeptides are labelled on the right hand side.

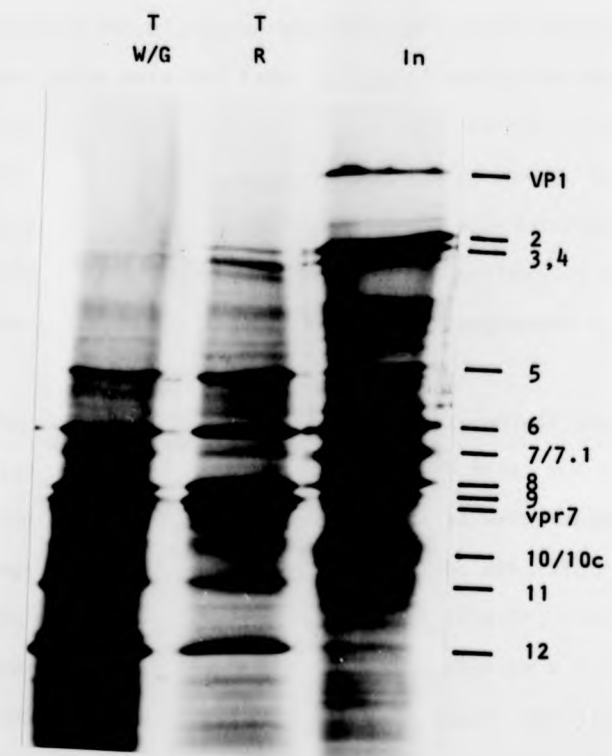
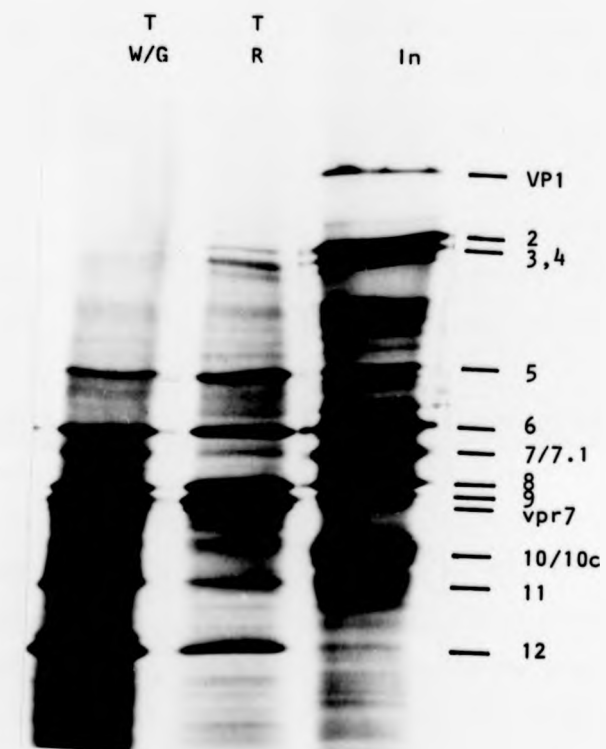


Fig.8 Comparison of the products of in vitro translation of rotavirus mRNA in the wheatgerm and rabbit reticulocyte lysate systems. A sample of the wheatgerm system (W/G) and the rabbit reticulocyte lysate (RR) were taken after in vitro translation (T) and compared to infected cells (In). The rotavirus-induced polypeptides are labelled on the right hand side.



4.5 (Cont'd.)

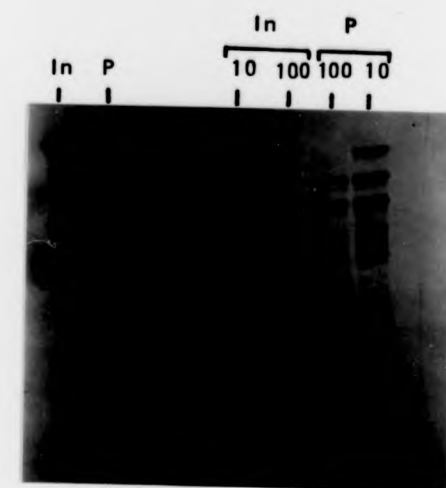
both reactions were analysed by PAGE in each case to compare the digestion patterns of any two proteins. Much more material from in vitro translation was needed than from infected cells to obtain the 10^6 c.p.m. needed for the peptide mapping, so the polypeptides from in vitro translation had to be immune precipitated (as described in chapter 5), to avoid overloading of gels when the translation products were separated by PAGE.

The peptides of VP1 and VP4 did not contain enough radioactivity for comparisons to be made, but all others from the two sources that comigrated were mapped. The peptide profiles from VP2, VP3, VP5, VP6, VP8, VP9 and VP12 revealed strong similarities (fig.9), confirming that the products of in vitro translation are indeed authentic viral polypeptides. However, profiles of VP2 and VP5 differ by one or more peptides. This difference in peptide profile could be due to:

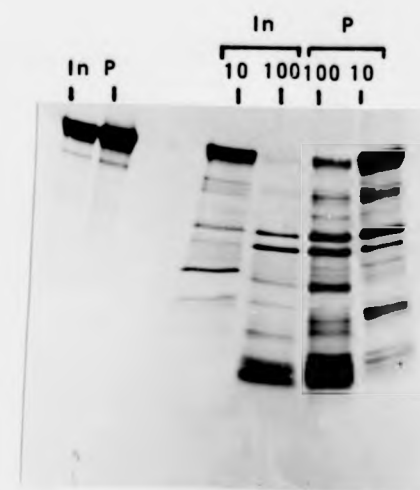
- (1) variation in the extent of digestion of the two polypeptides
- (2) a result from the passaging of the tissue culture adapted strain 17 times in LLCMK-2 cells and 5 times in BSC-1 cells after the virus which was purified from the faeces of an infected calf and used for in vitro transcription of our rotavirus mRNA
- (3) some minor contamination which has occurred in the cases of VP2 and VP9 with adjacent polypeptides.

Fig.9 Peptide maps of rotavirus proteins.

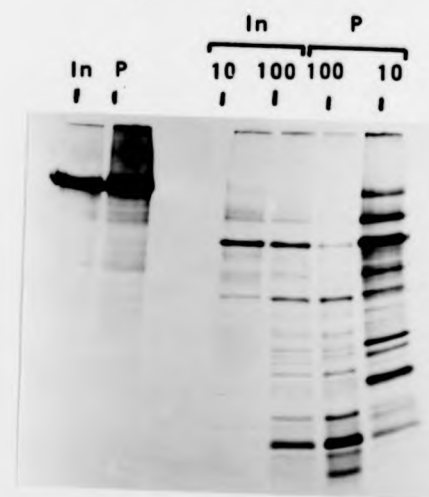
Proteins produced by in vitro translation (P) and in infected cells (In) were excised from polyacrylamide gels and digested with 10 or 100 $\mu\text{g/ml}$ S.aureus V8 protease as described in materials and methods. The undigested proteins and peptide profiles of a) VP2, b) VP3, c) VP5, d) VP6, e) VP8, f) VP9 and g) VP12 from the two samples are shown.



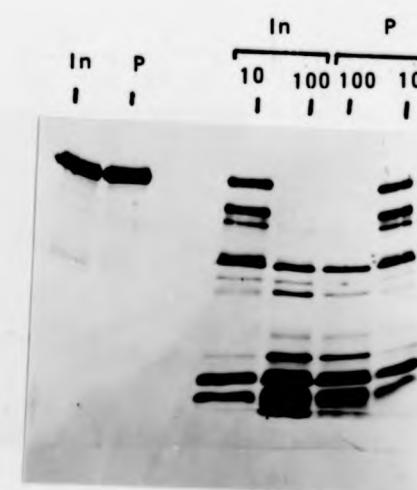
(a) VP2



(b) VP3



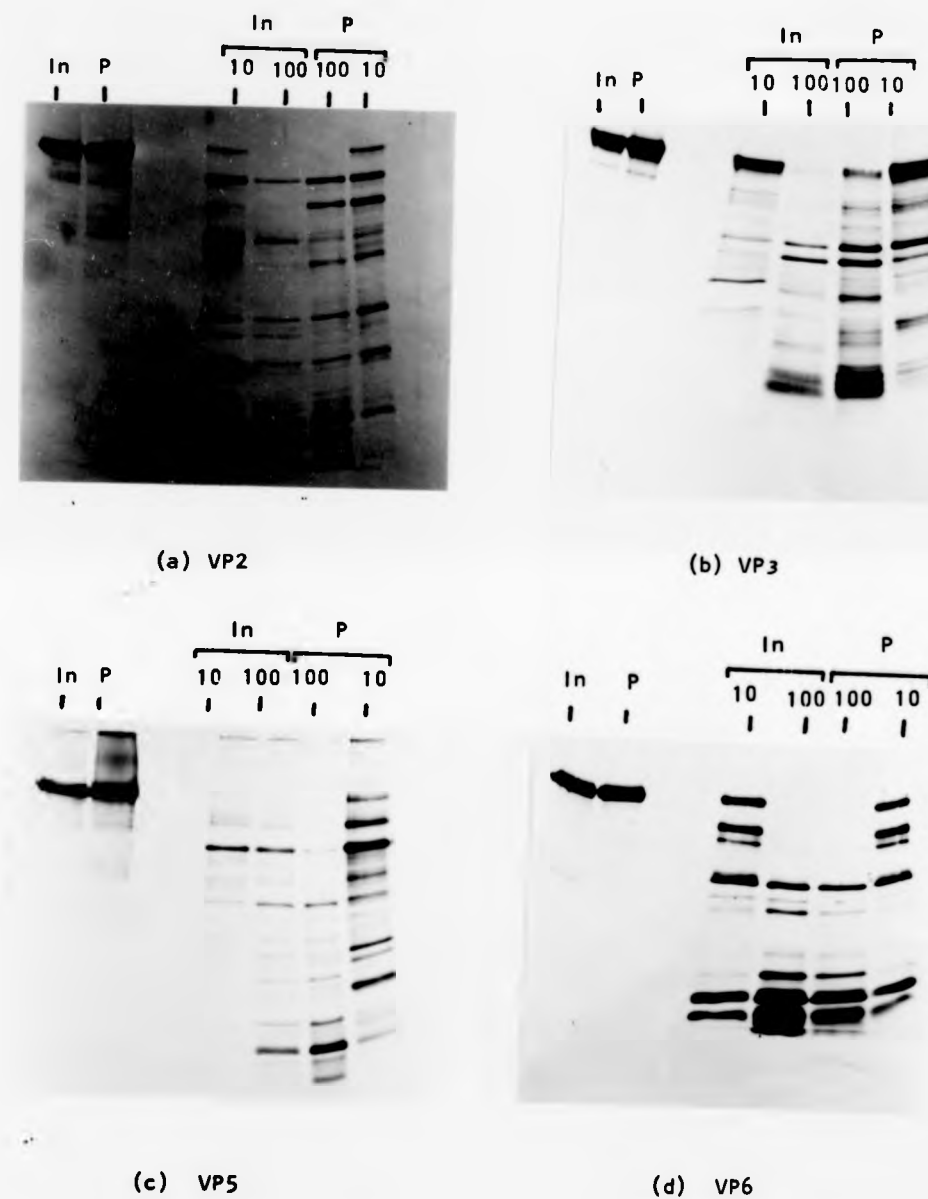
(c) VP5

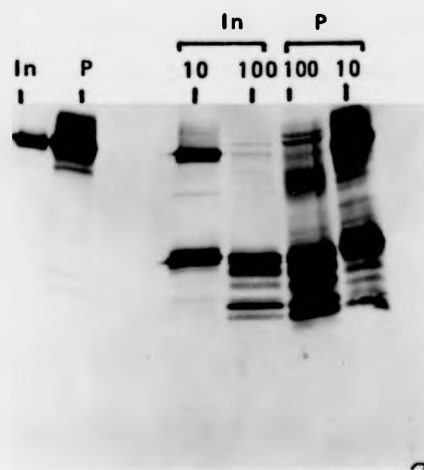


(d) VP6

Fig.9 Peptide maps of rotavirus proteins.

Proteins produced by in vitro translation (P) and in infected cells (In) were excised from polyacrylamide gels and digested with 10 or 100 $\mu\text{g/ml}$ S.aureus V8 protease as described in materials and methods. The undigested proteins and peptide profiles of a) VP2, b) VP3, c) VP5, d) VP6, e) VP8, f) VP9 and g) VP12 from the two samples are shown.

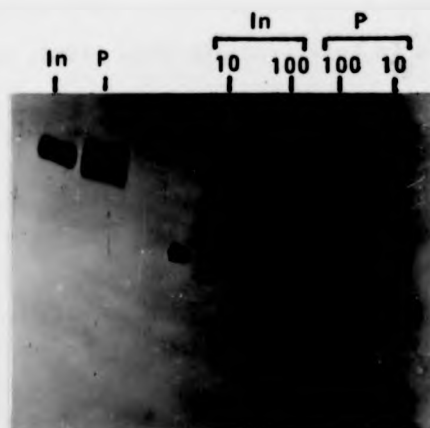




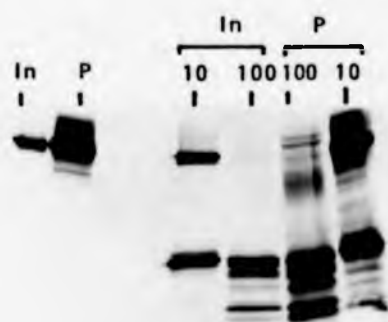
(e) VP8



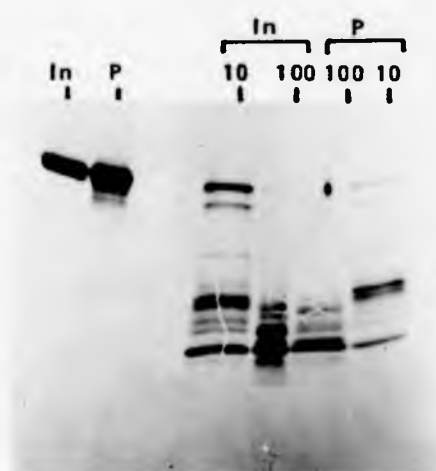
(f) VP9



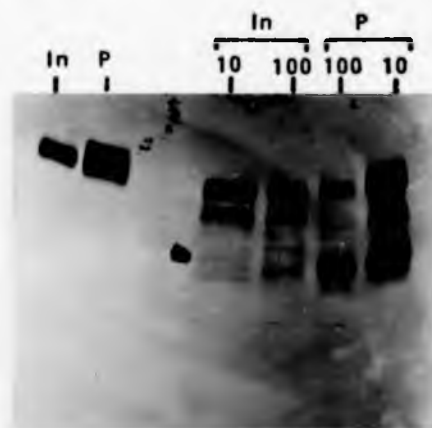
(g) VP12



(e) VP8



(f) VP9



(g) VP12

4.5 (Cont'd.)

(However, if contaminants were being detected one would expect to see several variant bands rather than just one) or (4) the fact that there are at least two different rotavirus polypeptides which comigrate with VP5. Therefore, the peptide maps have led us to conclude that all the polypeptides produced in vitro which comigrate with rotavirus proteins in infected cells are authentic rotavirus polypeptides.

4.6 Identification of rotavirus structural polypeptides.

The structural polypeptides of purified virus particles were identified by PAGE and polypeptides present in the inner and outer shells were distinguished by analysis of double shelled particles and single shelled particles. The structural polypeptides were radiolabelled by growing virus in the presence of ^{35}S -methionine, or were labelled in vitro with ^{125}I using the chloramine T method of Syvanen et al. (1973). Fig.10 compares the polypeptides of ^{35}S -methionine pulse-labelled infected cells with those of ^{35}S -methionine labelled purified virus. In the infected cell extracts, all the previously described polypeptides migrating between VP1 and VP10c are present, but in this case VP11 and VP12 were not seen. Three polypeptides of ^{35}S -methionine labelled virions obviously comigrate with polypeptides in infected cells - i.e. VP1, VP2 and VP6.

Fig.10 Rotavirus structural polypeptides.

- a) Single shelled (Ss) and double shelled (Ds) rotavirus particles grown in the presence of ^{35}S -methionine were compared to cells infected (In) with rotavirus at 10 p.f.u./cell and labelled with ^{35}S -methionine 6 to 6.5h pi and uninfected cells (Un) labelled at the same time. Numbering of the left refers to rotavirus polypeptides in infected cells and those on the right (centre piece) to structural polypeptides. Note VP7 and VP8 of Infected cells (arrowed) do not comigrate with VP7.1 and VP7.2 of virus particles.
- b) ^{125}I Ss and Ds particles are compared to ^{35}S -methionine labelled ds particles and to infected cells. Virus polypeptides are labelled on left (centre piece).

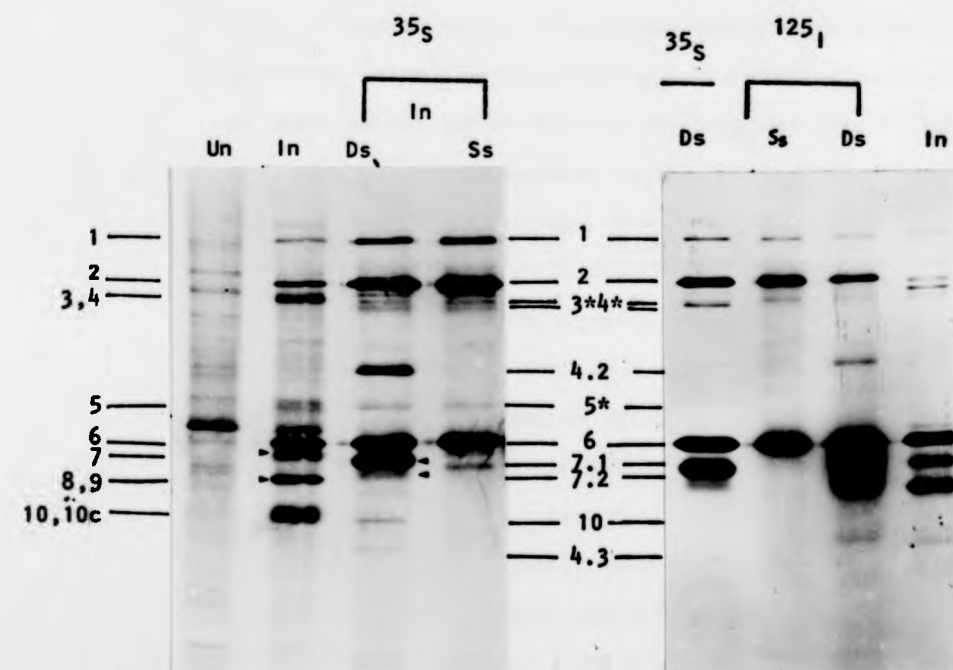
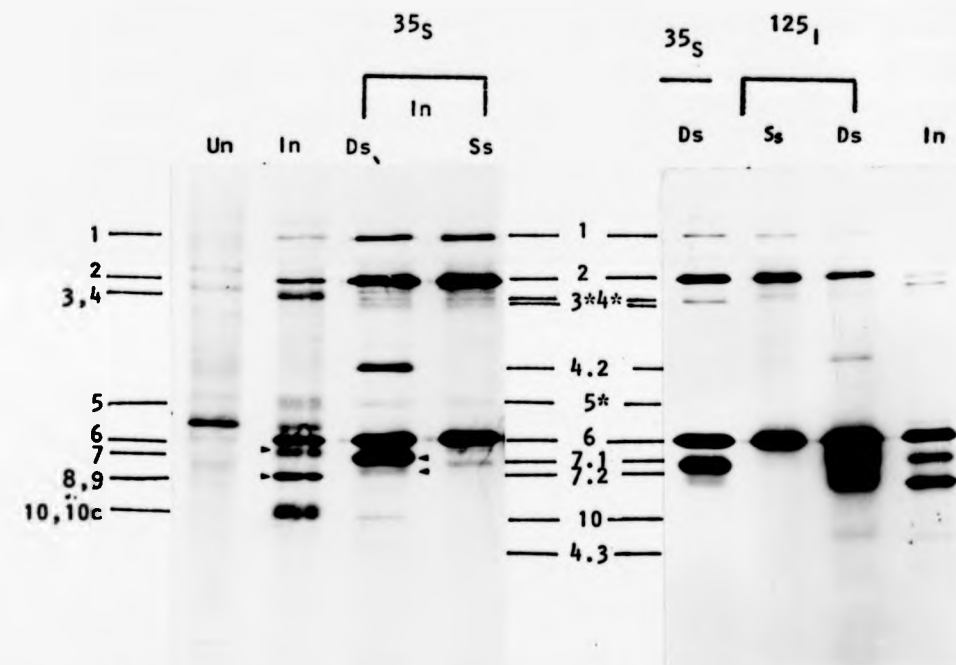


Fig.10 Rotavirus structural polypeptides.

- a) Single shelled (Ss) and double shelled (Ds) rotavirus particles grown in the presence of ^{35}S -methionine were compared to cells infected (In) with rotavirus at 10 p.f.u./cell and labelled with ^{35}S -methionine 6 to 6.5h pi and uninfected cells (Un) labelled at the same time. Numbering of the left refers to rotavirus polypeptides in infected cells and those on the right (centrepiece) to structural polypeptides. Note VP7 and VP8 of infected cells (arrowed) do not comigrate with VP7.1 and VP7.2 of virus particles.
- b) ^{125}I Ss and Ds particles are compared to ^{35}S -methionine labelled ds particles and to infected cells. Virus polypeptides are labelled on left (centrepiece).



4.6 (Cont'd.)

These three proteins are also the major polypeptides of the ^{35}S -methionine single shelled particle. Single shelled particles also contain minor proteins which do not comigrate with any protein in infected cells. These are VP3*,4* and VP5* and are discussed below. Double shelled particles contain several extra polypeptides compared to single shelled particles which are designated outer shell polypeptides. Many of these do not comigrate with polypeptides seen in infected cells and are thus thought to be the result of further post-translational modification. The outer shell proteins are VP4.2, VP7.1, VP7.2, VP10c and VP4.3. The major outer shell protein is VP7.1, which migrates just below VP7 of infected cells and comigrates with its natural cleavage product VP7.1, sometimes found in infected cells (section 4.1). VP7.2, migrating just faster than VP7.1, has been shown by peptide mapping to be a close relation of VP7.1 with a single peptide lost (Espejo et al., 1981). VP7.1 was called VP7c by McCrae and Faulkner-Valle (1981) and VP7 by Espejo et al. (1981) and Estes et al. (1981). VP7.2 was called VP7a by Espejo et al. (1981) and Estes et al. (1981) and VP8 by McCrae and Faulkner-Valle (1981), because it appeared to migrate with VP8 of infected cells. However, on close examination it can be seen that, while VP7.2 migrates close to VP8, it does not comigrate with it and so VP8 is now designated non-structural. VP10c is a minor outer shell protein migrating below VP7.2 and is a natural cleavage product

4.6 (Cont'd.)

of VP10. Both VP10 and VP10c are seen in infected cells. VP10c in virus particles is normally just termed VP10. Polypeptide VP4.2, which migrates between VP3 and VP5 of infected cells, is only seen in virus which has been in the presence of trypsin and it has been shown to be a trypsin cleavage product of VP3 by Estes et al. (1981). VP4.3 is a second cleavage product of VP3 but we never see as much of it as of VP4.2, and we conclude that it is further degraded by residual chymotrypsin in our trypsin preparation, as Estes et al. (1981) showed that VP4.3 is degraded by chymotrypsin treatment. The three minor proteins VP3*,4* and VP5*, seen in both single shelled and double shelled particles are also a result of proteolytic cleavage. They are trypsin cleavage products of VP2 and are thus unrelated to VP3, VP4 or VP5, although they migrate very close to them (Estes et al., 1981).

Fig. 10 also shows radiolabelled proteins of iodinated (^{125}I -labelled) virus in which only polypeptides exposed on the virus surface should have been labelled. Both double shelled and single shelled particles were examined. In both cases all the virus polypeptides identified in ^{35}S -methionine labelled virus became labelled with ^{125}I , suggesting that none were hidden from the virus surface. This unexpected result was also reported by Novo and Esparza (1981) and R. Saunders (personal communication).

4.6 (Cont'd.)

Use of the lactoperoxidase method for ^{125}I -labelling, which is reported to be milder, gave the same result. Some of the minor outer shell proteins such as VP7.2 and VP10 were more strongly labelled by iodination than by ^{35}S -methionine labelling (see also fig. 6.1), so this method was useful for locating minor polypeptides, as they are used in chapter 6. ^{125}I -labelled double shelled particles did not only contain radiolabelled outer shell proteins, but also inner shell proteins, suggesting that either the inner shell proteins are not completely masked by the outer shell or that, as suspected, our preparation also contained some single shelled particles.

Rotavirus polypeptides have also been observed by staining protein in gels with Coomassie brilliant blue (fig.11). Again the same polypeptides were identified and their relative intensities appeared to be comparable to proteins observed in ^{35}S -methionine labelled virus, except that VP7.1 and VP7.2 have apparently lower intensity in the stained preparations. Two polypeptides are seen in the stained preparation which are never detected in ^{35}S -methionine labelled virus:- X migrates above VP4.2 and Y migrates below VP7.1. These are thought to be contaminating proteins associated with virus particles.

Fig.11 Structural proteins of calf rotavirus
visualised by Coomassie blue staining.

The proteins of single shelled (Ss) and double shelled (Ds) particles were separated by PAGE and stained with Coomassie brilliant blue. Two photographs are shown in fig.11 as a) shows high molecular weight polypeptides better and b) shows low molecular weight polypeptides better. The polypeptides are labelled on the left hand side of each photograph.

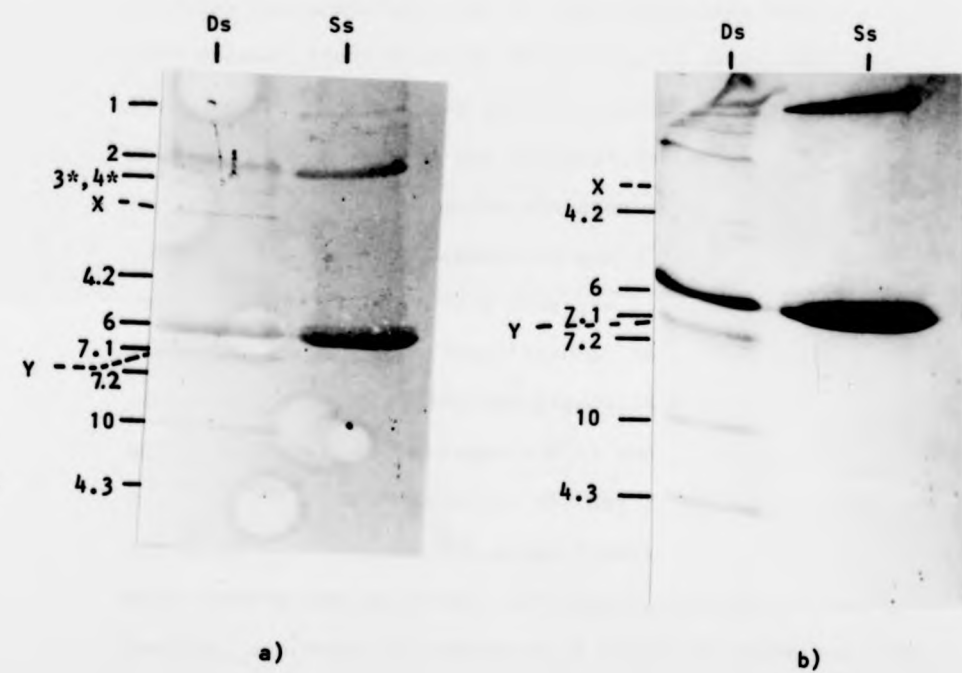
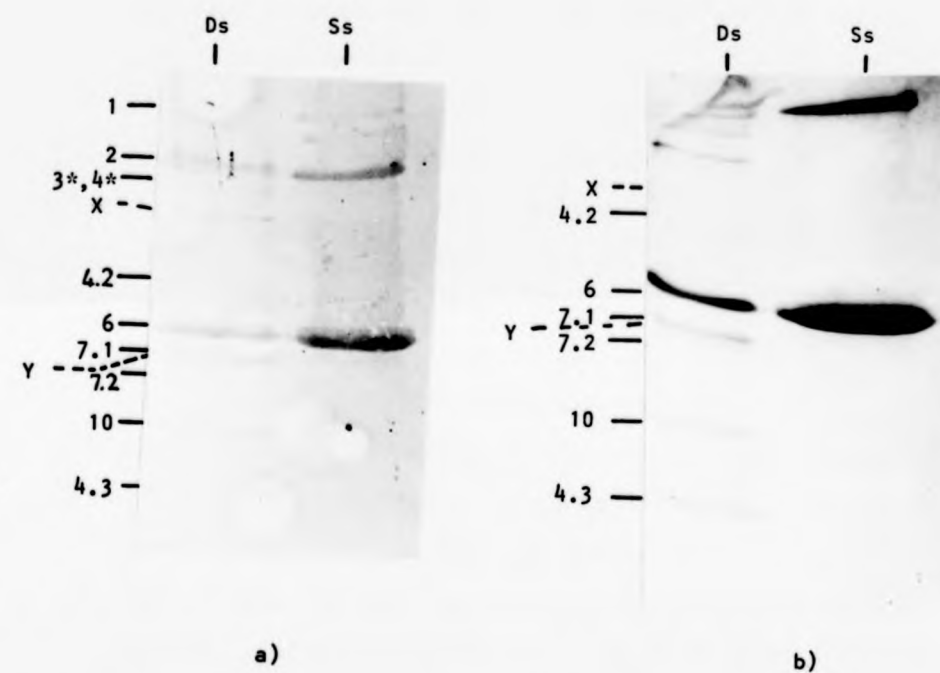


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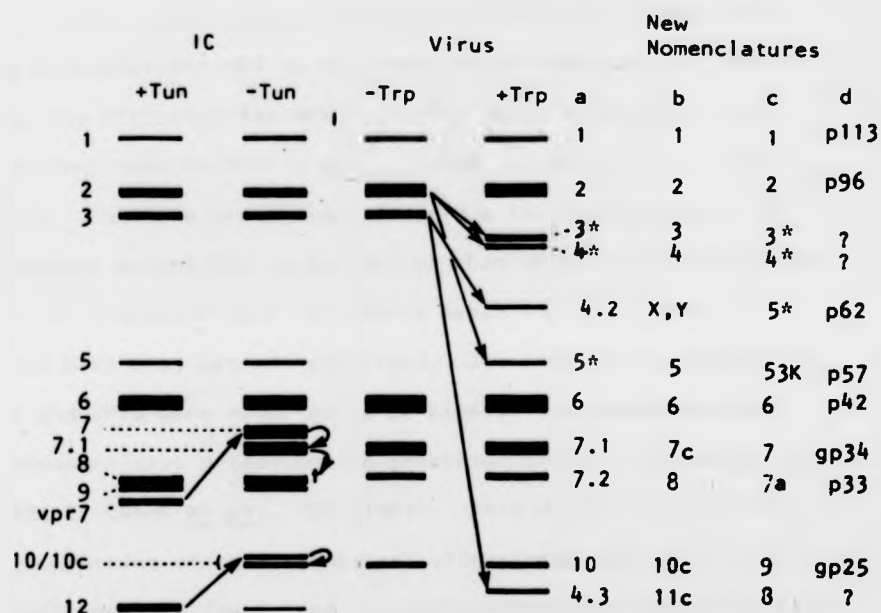
CHAPTER 4

DISCUSSION

From the experiments described in this chapter and from concomitant published work by other groups, we have been able to build a model for the production and processing of rotavirus proteins. Use of high resolution gradient gels allowed identification of previously uncharacterised proteins. However, there are still many unanswered questions about the processing of the polypeptides. Many of the primary gene products, which are seen after in vitro translation and also in tunicamycin-treated infected cells, are extensively modified before they are assembled into infectious virus particles. Post-translational modifications of the primary gene products include glycosylation, natural cleavage and trypsin-induced cleavage and it should also be mentioned that natural heterogeneity in VP7 has recently been reported (Estes et al., 1982). The steps involved in modification of each protein are described individually in the results section, and a model is presented in fig.12 to summarise them.

Structural proteins VP1 and VP6 and non-structural proteins VP4, VP5, VP8 and VP9 do not appear to be post-translationally modified enough to alter their migration in PAGE. All the other primary gene products - VP2, VP3, vpr7 and VP12 - become modified and, with the possible exception of VP11, most of the rotavirus structural proteins are formed from these precursors.

Fig.12 Summary of the modification steps involved in the maturation of rotavirus proteins



Current status of the nomenclature and processing of rotavirus polypeptide (VP) in infected cells and virus particles. Arrows indicate putative processing steps through proteolysis or glycosylation which have been demonstrated by treatment of virus with trypsin (Trp) or of infected cells with tunicamycin (Tun) respectively. Note that VP7.2 in infected cells (+) is only seen after immune precipitation with VP7.1 or VP7.2 as it is obscured by the more abundant VP8. The nomenclature used in this report is indicated under heading (a), that of McCrae and Faulkner-Valle (1981) in (b), that of Estes *et al.* (1981) in (c) and that of Dyall-Smith and Holmes (1981b) in (d). In our nomenclature we use * to indicate cleavage products of VP2.

vpr7 and VP12 are not seen in infected cells and two proteins of somewhat higher molecular weight replace them. Comparison of infected cells in the presence and absence of tunicamycin reveals that both become glycosylated, forming structural proteins VP7 and VP10. We have been able to show using mono-specific antisera (chapter 6, Killen and Dimmock, 1982) that the precursor for VP7 is vpr7 and, by elimination, we infer that the precursor for VP10 is VP12. This result has since been confirmed by Sabara et al. (1982) using limited proteolysis. The same group also reported a third glycoprotein of molecular weight 16K (i.e. smaller than VP12) but this protein and its precursor were very faint bands and its presence in virus particles was not confirmed. The carbohydrate chains of VP7 and VP10 were reported to be cleavable by endo- β -acetylglucosaminidase H leaving the precursor protein (Sabara et al., 1982; Erickson et al., 1982, 1983). This enzyme is specific for glycoproteins containing neutral oligosaccharides with a core of Asn-(GlcNAc)₂ - (Man), and a subsequent mannose-rich chain, but does not attack more complex oligosaccharides with the same core or the types of glycoproteins processed by golgi (Tarentino and Maley, 1974). Thus, the simian rotavirus glycoproteins appear to contain exclusively 'high mannose' carbohydrates (Erickson et al., 1983). Then VP7 and VP10 both become modified further by natural cleavage before assembly into virus particles (fig.12). A small piece of VP10 is cleaved off to form the structural protein VP10c. VP7 is more extensively cleaved to VP7.1 and VP7.2. The method of production of VP7.2 is highly questionable at present,

but VP7.2 has been shown to have the same peptide profile as VP7.1 with at least one peptide obviously missing (Espejo et al., 1981). We have confirmed their relationship using our monospecific antisera (chapter 6, Killen and Dimmock, 1982). Alternative models for production of VP7.2 are shown in fig.13. Estes et al. (1982) reported heterogeneity of VP7 among simian rotaviruses but they did not extend this to determine if VP7.1 and VP7.2 also show heterogeneity. This raises the question of whether VP7.1 and VP7.2 are heterogeneous forms of a single structural polypeptide (each found in a different virus particle) formed by model 13c. However, although we always see VP7.1 and VP7.2 in virus particles, we usually only see one band corresponding to VP7 in infected cells (see also gels in chapters 5 and 6). When two bands are seen in infected cells, the faster migrating band is stronger and comigrates with VP7.1. This is incompatible with formation of VP7.1 from the larger and VP7.2 from the smaller of two forms of VP7, as VP7.1 is the major constituent in virus particles. Each variant form of VP7 is said to be stable for at least ten passages (Estes et al., 1982) and our virus was plaque purified three times and passaged less than five times subsequently. VP7.2 is therefore more likely to be formed by either model a) or b) than by model c).

Fig. 13

Alternative models for formation of VP7.1 and VP7.2



Not all publications have recognised the presence of VP10 in virus particles, as it is a very weak band (fig.10). However, we have raised a monospecific antiserum against it and this serum neutralizes rotavirus infectivity (chapters 6 and 7, Killen and Dimmock, 1982). Bastardo et al. (1981) also have monospecific antisera against VP10. Therefore, this polypeptide must be a constituent of rotavirus particles. There is also some doubt about whether VP11/11c is a constituent of virus particles as reported by McCrae and Faulkner-Valle (1981). We see a minor polypeptide migrating below VP10 but cannot determine if it comigrates with VP11 as this protein is rarely seen in infected cells. We have called this structural polypeptide VP4.3, as VP4.3 is well described (Espejo et al., 1981; Estes et al., 1981; Clark et al., 1981) and we see no other virus protein which could be VP4.3.

VP2 and VP3 are both cleaved by trypsin (Espejo et al., 1981; Estes et al., 1982), which is known to greatly enhance rotavirus infectivity (Almeida, 1978). All of VP3 is cleaved to VP4.2 and VP4.3 (fig.12), and this process is thought to be the mechanism of the enhancement of infectivity. In particular, VP4.2 is thought to be the important product for infectivity as some VP4.3 is further cleaved by other proteolytic enzymes without destroying infectivity (Estes et al., 1981). VP3 is the product of gene segment 4, which determines

restriction of growth in tissue culture (Greenberg et al., 1983). It is interesting to note that immune precipitation of infected cells in chapter 5 revealed at least 4 bands in the region of VP4.2, but only one is incorporated into virus particles so VP4.2 may also be formed in several stages. A small amount of VP2 is also cleaved by trypsin, forming products VP3*,4* and VP5*, but the majority of VP2 does not become cleaved (Estes et al., 1981). There is no evidence to suggest that this cleavage step is necessary or important.

It was interesting and surprising to find that ^{so many} rotavirus proteins are so extensively processed before they appear in mature virus particles since in reovirus most of the structural proteins are primary gene products (Both et al., 1975). The exception is the major outer shell protein $\mu 1$ which is glycosylated (Krystal et al., 1976) and some of it is cleaved to $\mu 1c$ (Joklik, 1981). $\mu 1$ has also been shown to be phosphorylated (Krystal et al., 1975) and polyadenylated (Carter et al., 1980). Neither of these latter modifications have been investigated with rotavirus polypeptides. Rotavirus also differs from reovirus in that it requires proteolytic enhancement - reoviruses are naturally infectious, although their infectivity is slightly enhanced by pancreatin (Wallis et al., 1966). The reovirus transcriptase is activated by chymotrypsin in vitro which

removes $\sigma 1$ and $\sigma 3$ from the outer shell and removes or cleaves $\mu 1c$, depending on the ionic strength (Joklik, 1981). However, chymotrypsin is not necessary for infectivity in vivo as the reovirus particle is naturally converted to a subviral particle by lysosomal enzymes after uptake into the cells (Silverstein et al., 1976).

CHAPTER 5

CHAPTER 5

Identification of rotavirus antigens and the determination of immunological cross-reactivity of rotavirus polypeptides from different serotypes.

Introduction: The existence of group-specific, subgroup-specific and type-specific rotavirus antigens has been recognised (Thouless et al., 1977; Bridger, 1978; Kapikian et al., 1981), but the antigenic specificity or function of any individual polypeptide has not been determined.

The rotavirus group-specific antigens are those found in all the rotaviruses of a single group (e.g. of group 1), which will react with antisera against any virus in that rotavirus group but will not react at all with antisera against the other rotavirus groups (e.g. the new groups) or with antisera against the other genera of the reovirus family. At least some of the antigens reacting by immunofluorescence are group-specific. The subgroup-specific antigens are those which are common to all members of the same subgroup but will not react with antisera against other subgroups or groups. The antigen(s) detected by ELISA are subgroup-specific. The type-specific antigens are only found in one rotavirus serotype and will not react with antisera against other serotypes. An example is the neutralization-specific antigen.

In this section, immune precipitation of radiolabelled rotavirus proteins from infected cells was used to identify the rotavirus antigens and distinguish group-specific from type-specific antigens as, under ideal conditions, individual proteins can be specifically precipitated from a mixture by the appropriate antibody. This chapter provides a preliminary characterisation of the rotavirus antigens as an introduction to experiments described in chapters 6 and 7 in which antigens are further characterised by their reaction with monospecific antisera raised against them. A monospecific antiserum is raised against a single protein and is specific for antigenic determinants found on that protein.

5.1 Optimisation of the specificity and efficiency of the immune precipitation.

A preliminary indication of specificity was seen by determining if virus polypeptides could be specifically precipitated from samples in which both host and virus proteins were labelled with ^{35}S -methionine. Immune

5.1 (Cont'd.)

precipitation using the original Kessler (1975) method in which proteins were precipitated in the presence of 0.5% NP₄₀ always resulted in some non-specific precipitation, so 'high detergent' buffer as described in the methods section was substituted and this modification greatly improved the specificity and reproducibility of the reaction. Other modifications of the original method include use of a relatively large volume for the reaction (i.e. 10 µl serum and 10 µl cell extract were diluted to 500 µl) and precipitating at 4°C overnight with stirring, which improved the efficiency of precipitation.

The percentage of TCA-precipitable counts recovered after immune precipitation was also monitored. In samples of rotavirus polypeptides translated in vitro in rabbit reticulocyte lysates, 20% and 11% of the total TCA precipitable radioactivity was precipitated from by the original and modified methods respectively. 5-10% of the total TCA precipitable radioactivity from cells infected with rotavirus at 5 p.f.u./cell and labelled 6-6½h p.i. was routinely recovered by immune precipitation with 'high detergent' buffer.

Purification of immunoglobulin from serum by protein A-Sepharose chromatography, so that antibodies which could bind to rotavirus proteins but not to protein A were

5.1 (Cont'd.)

eliminated, did not increase either the efficiency or specificity of precipitation.

Sometimes spontaneous formation of precipitates in the detergent buffer occurred and precautions to prevent this were necessary. The first was careful removal of membranous material from the original cell extracts by treatment of lysed cells with 0.5% NP₄₀ in hypotonic buffer followed by Dounce homogenisation and centrifugation. Use of this extract also improved the efficiency of precipitation. Another precaution was to dilute the cytoplasmic extract in detergent buffer and then centrifuge to pellet spontaneous precipitates, before addition of serum. Freshly prepared detergent buffer was also important for specific immune precipitation.

5.2 Demonstration of the specificity of the immune precipitation reaction.

Elimination of host protein bands from infected cell extracts was preliminary confirmation of the specificity of immune precipitation. A further test of specificity was taken by mixing cytoplasmic extracts with ¹²⁵I-labelled influenza virus and immune precipitation with anti-rotavirus convalescent serum (G203). Rotavirus polypeptides were specifically precipitated, while the ¹²⁵I influenza virus proteins were not detectable in the immune precipitates (fig.1).

5.2 (Cont'd.)

Fig.1 also demonstrates that rotavirus polypeptides are specifically selected from cells infected with a low multiplicity (1 p.f.u./cell) of rotavirus in which the rotavirus polypeptides are barely visible.

5.3 Optimisation of conditions for immune precipitation.

5.3.1 Infected Cells.

The cell extracts chosen for immune precipitation had prominent radiolabelled viral polypeptides as well as host proteins. At an m.o.i. of 0.1 and 1 p.f.u./cell the synthesis of virus polypeptides was too weak for detection, while a multiplicity of 20 p.f.u./cell caused total shut-off of host protein synthesis. Multiplicities of 5-10 p.f.u./cell produced cells showing both radiolabelled virus and host proteins. A multiplicity of 5 p.f.u./cell pulse labelling with ^{35}S -methionine at 6-6½h p.i. were chosen as standard conditions. However, even under standardised conditions, some variations in the quantity of individual polypeptides was detectable, probably due to variation in health, age and confluence of the cells. In particular, the amount of VP12 processed to VP10 and the quantities of VP7 and VP7.1 varied. VP4.2 was only seen by immune precipitation and the quantity of this polypeptide also varied.

Fig.1 Demonstration of the specificity of immune precipitation.

^{125}I -labelled influenza virus was mixed with cells infected with calf rotavirus at 5 p.f.u./cell and labelled with ^{35}S -methionine 6-6½h p.i. and the mixture was immune precipitated with calf rotavirus serum (C1). Cells infected with calf rotavirus at 1 p.f.u./cell were also immune precipitated. Nomenclature of rotavirus polypeptides is shown on the right hand side and that of external influenza virus polypeptides on the left.

I = ^{125}I labelled Influenza (A/FPV/Rostok/34) virus.
 RI = ^{125}I labelled influenza virus mixed with cells infected with 5 p.f.u./cell rotavirus. R2 = cells infected with rotavirus at 1 p.f.u./cell. IP = immune precipitate of RI. IP2 = immune precipitate of R2.

HA1 and HA2, the two polypeptides of influenza virus haemagglutinin are labelled.

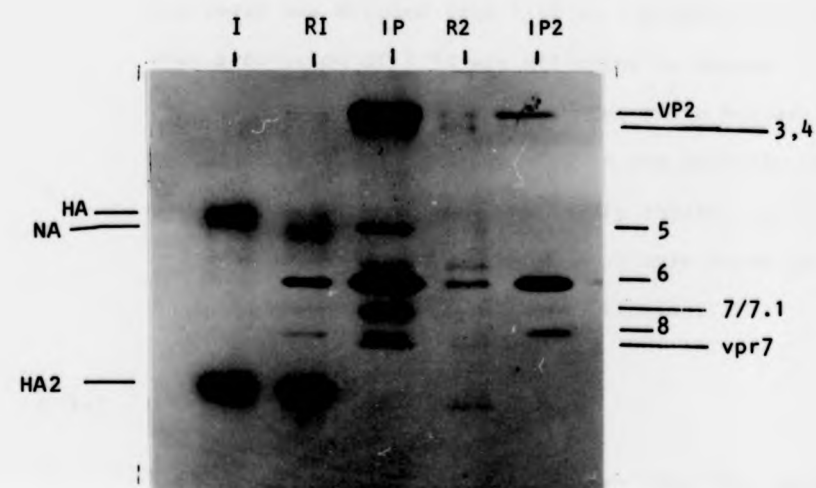


Fig.2 Optimisation of antiserum concentration

Immune precipitation of cells infected with calf rotavirus at 5 p.f.u./cell and labelled 6-6½h p.i. was carried out with C1 antiserum diluted at 1. 1/50 2. 1/500 3. 1/5,000 4. 1/50,000 5. 1/500,000 and 6. 1/1,000,000 and the immune precipitates were analysed by PAGE. Conditions of the experiment are described in Fig.1.

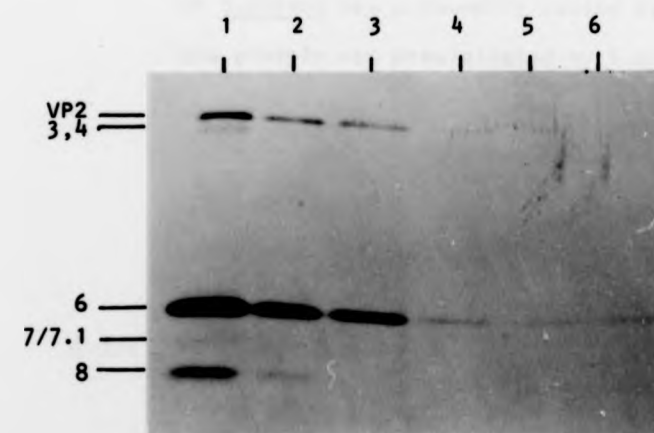


Fig.1 Demonstration of the specificity of immune precipitation.

^{125}I -labelled influenza virus was mixed with cells infected with calf rotavirus at 5 p.f.u./cell and labelled with ^{35}S -methionine 6-6½h p.i. and the mixture was immune precipitated with calf rotavirus serum (C1). Cells infected with calf rotavirus at 1 p.f.u./cell were also immune precipitated. Nomenclature of rotavirus polypeptides is shown on the right hand side and that of external influenza virus polypeptides on the left.

I = ^{125}I labelled influenza (A/FPV/Rostok/34) virus.
 RI = ^{125}I labelled influenza virus mixed with cells infected with 5 p.f.u./cell rotavirus. R2 = cells infected with rotavirus at 1 p.f.u./cell. IP = immune precipitate of RI. IP2 = immune precipitate of R2.
 HA1 and HA2, the two polypeptides of influenza virus haemagglutinin are labelled.

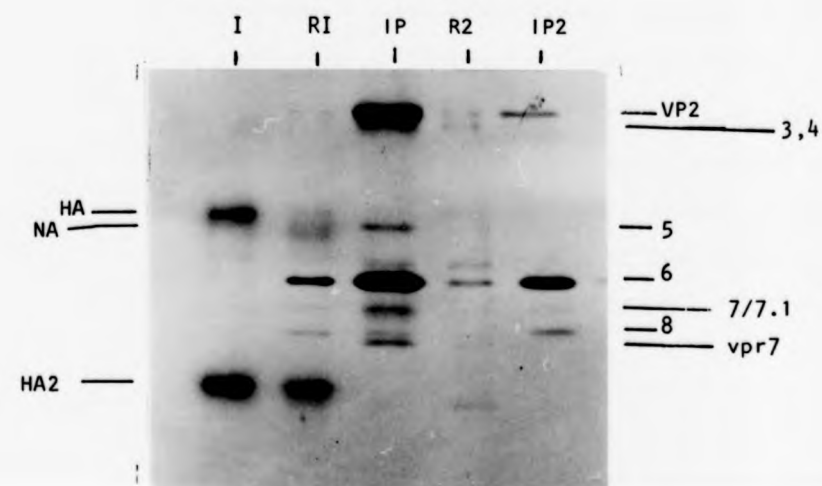
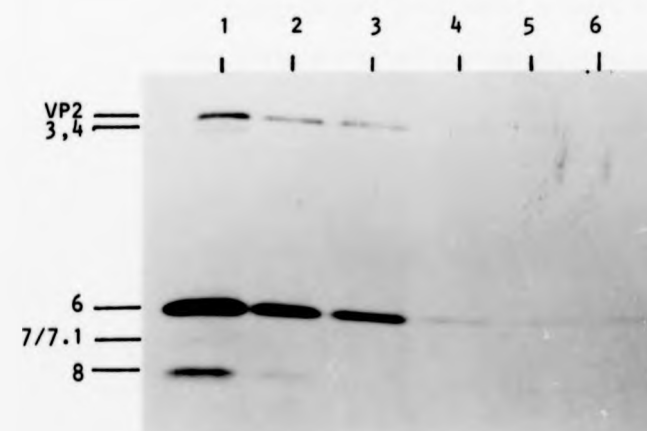


Fig.2 Optimisation of antiserum concentration

Immune precipitation of cells infected with calf rotavirus at 5 p.f.u./cell and labelled 6-6½h p.i. was carried out with C1 antiserum diluted at 1. 1/50 2. 1/500 3. 1/5,000 4. 1/50,000 5. 1/500,000 and 6. 1/1,000,000 and the immune precipitates were analysed by PAGE. Conditions of the experiment are described in Fig.1.



5.3.2 Antiserum concentration.

Antiserum was diluted from $1/50$ to $1/4000000$. Fig.2 shows that a dilution of $1/50$ was efficient in immune precipitating rotavirus polypeptides while higher dilutions resulted in a decrease in the quantity of antigen precipitated. As previously stated, purified immunoglobulin was no more efficient than serum in immune precipitation at the same dilution.

5.3.3 Concentration of S.aureus.

The concentration of S.aureus was an important parameter - the optimum lay in the range of 10-50 μ l of a 10% suspension in a final volume of 500 μ l and a greater or lesser concentration resulted in decreased precipitation (fig.3). Decreased precipitation by high concentrations of S.aureus was presumably caused by 'trapping' - i.e. the protein was precipitated by S.aureus but the solubilisation of this protein was inhibited by the large bacterial pellet, at least under the conditions used for these experiments. 25 μ l of S.aureus was used in subsequent experiments.

5.3.4 pH.

The pH of the reaction in the range pH7.4 to pH9.0 did not significantly affect the results (fig.4) - the suggested pH of 8.2 was therefore taken as optimum.

Fig.3 Optimisation of *S.aureus* concentration

Cells infected with 5 p.f.u./cell calf rotavirus and labelled 6-6½h p.i. were immune precipitated with CI antiserum. Immune complexes were precipitated with 1. 0.1 µl 2. 1 µl 3. 5 µl 4. 10 µl 5. 50 µl 6. 100 µl and 7. 500 µl of a 10% solution of formalin-fixed *S.aureus*. In = non-precipitated infected cells.

Fig.4 Optimisation of pH

Immune precipitation of cells infected with 5 p.f.u./cell calf rotavirus and labelled 6-6½h p.i. was carried out with CI antiserum at the pH values indicated. In = infected cells.

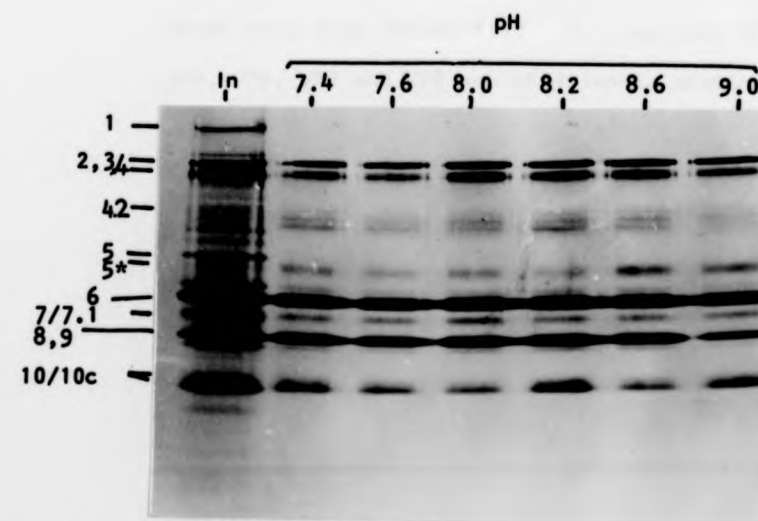
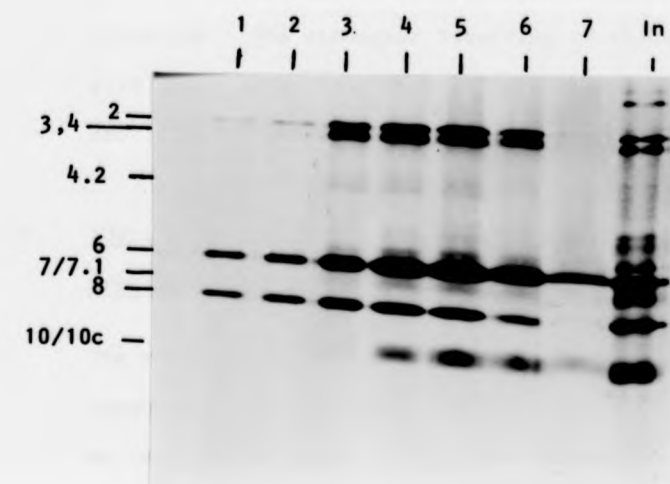


Fig.3 Optimisation of *S.aureus* concentration

Cells infected with 5 p.f.u./cell calf rotavirus and labelled 6-6½h p.i. were immune precipitated with CI antiserum. Immune complexes were precipitated with 1. 0.1 µl 2. 1 µl 3. 5 µl 4. 10 µl 5. 50 µl 6. 100 µl and 7. 500 µl of a 10% solution of formalin-fixed *S.aureus*. In = non-precipitated infected cells.

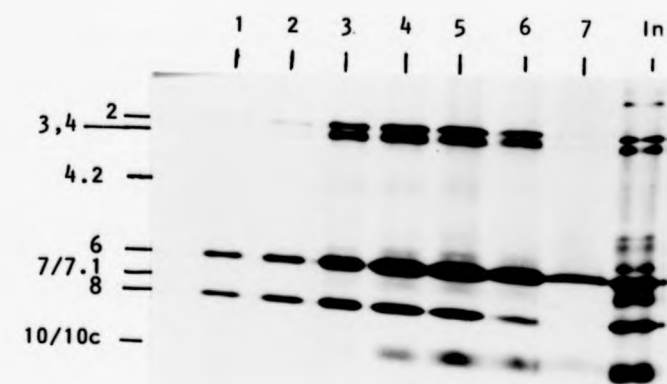
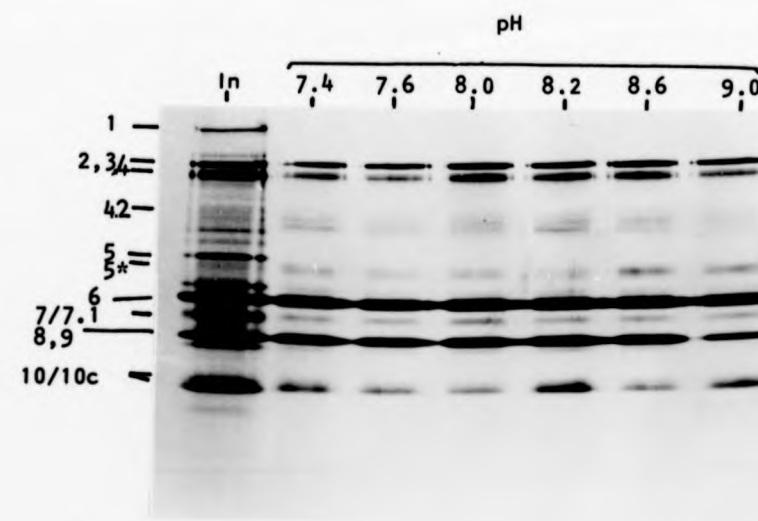


Fig.4 Optimisation of pH

Immune precipitation of cells infected with 5 p.f.u./cell calf rotavirus and labelled 6-6½h p.i. was carried out with CI antiserum at the pH values indicated. In = infected cells.



5.3.5 Determination of the optimum time to label infected cells.

A time course of immune precipitation is shown in fig.5 - precipitation is best from cells labelled 6-8h after infection. The strongest labelling of virus protein was also shown to be at this time (see chapter 4). Cells were routinely labelled at 6-6½h for immune precipitation.

5.4 Identification of rotavirus polypeptides immune precipitated from infected cells.

The rotavirus antigens in infected cells reacting with homologous convalescent anti-rotavirus serum (CI) were identified by immune precipitation and PAGE of the precipitates. The products of immune precipitation are compared to infected cells in fig.6. Most of the host polypeptides are absent in the immune precipitates, and some viral bands have been intensified. Polypeptides VP2, VP3 and VP4, VP6, VP7 and VP8 are efficiently precipitated. The faster migrating VP3 is precipitated much more strongly than VP4. VP11 is a faint band in both the infected cells and in the immune precipitates. VP1 was not precipitated and VP10/10c were inefficiently precipitated as the immune precipitated polypeptides appeared less intense than the polypeptides in the original sample when equal c.p.m. of both samples were loaded. Precipitation of VP10 is shown later to be variable, e.g. it is precipitated (to varying degrees) in figs. 3, 4, 6, 8 and

Fig.5 Time course of protein synthesis in infected cells detected by immune precipitation.

Cells were infected with 5 p.f.u./cell calf rotavirus and labelled with ^{35}S -methionine for 30 min at the times shown. Equal c.p.m. of each sample were immune precipitated and each precipitate was analysed by PAGE.

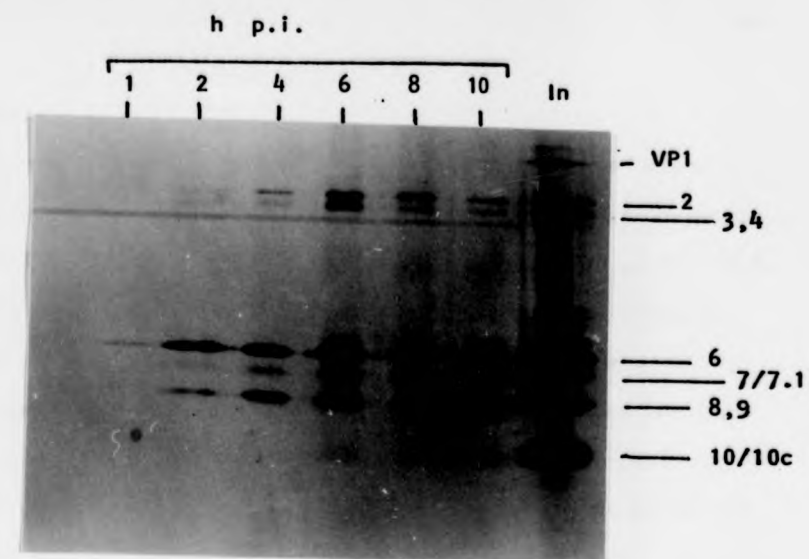


Fig.6 Immune precipitation of polypeptides from calf rotavirus-infected cells.

Calf rotavirus-infected cells were immune precipitated under optimum conditions and the immune precipitates analysed by PAGE. In = infected cells (5 p.f.u./cell), labelled 6-6½h p.i. IP = immune precipitate using anti-calf rotavirus serum C1. Numbers on the left hand side refer to polypeptides seen in infected cells and those on the right refer to polypeptides not detected by PAGE of infected cell extracts but detected in immune precipitates of these extracts.

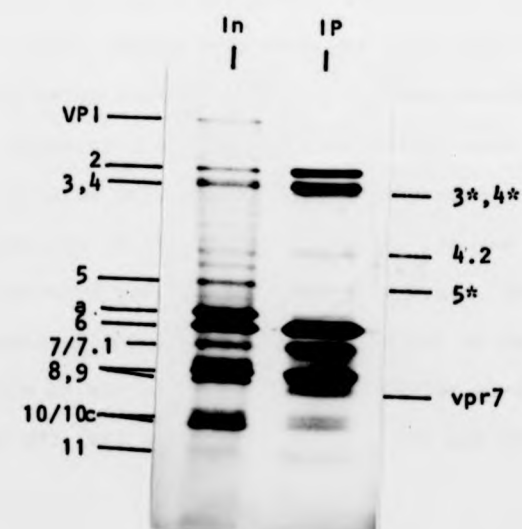


Fig.5 Time course of protein synthesis in infected cells detected by immune precipitation.

Cells were infected with 5 p.f.u./cell calf rotavirus and labelled with ^{35}S -methionine for 30 min at the times shown. Equal c.p.m. of each sample were immune precipitated and each precipitate was analysed by PAGE.

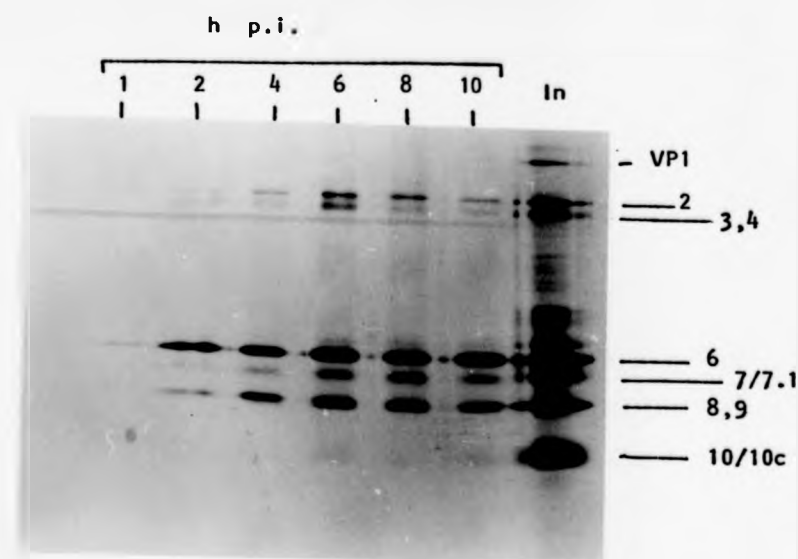
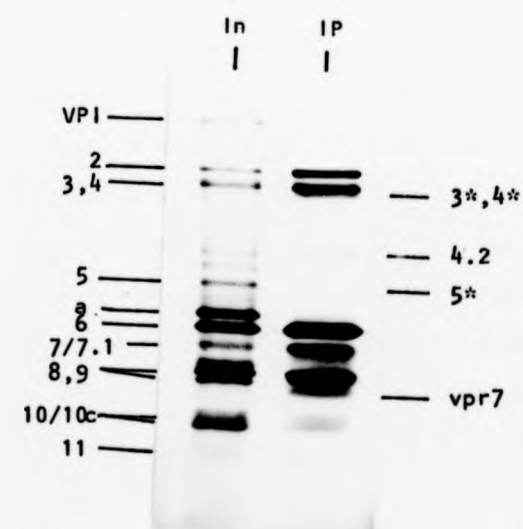


Fig.6 Immune precipitation of polypeptides from calf rotavirus-infected cells.

Calf rotavirus-infected cells were immune precipitated under optimum conditions and the immune precipitates analysed by PAGE. In = infected cells (5 p.f.u./cell), labelled 6-6½h p.i. IP = immune precipitate using anti-calf rotavirus serum C1. Numbers on the left hand side refer to polypeptides seen in infected cells and those on the right refer to polypeptides not detected by PAGE of infected cell extracts but detected in immune precipitates of these extracts.



5.4. (Cont'd.)

some of 10 but not in figs. 1, 2, 5, 7 and 9. The immune precipitate of VP9 is masked by VP8 and VP12 is not observed in this case. Where VP9 is resolved from VP3 - e.g. in fig.4, it is inefficiently precipitated. VP4.2 is not visible by PAGE of extracts from infected cells but it can be seen in immune precipitates of these extracts (as more than one band - see also figs. 3 and 4). The same is true of the putative vpr7 and a band (possibly VP3*, 4*) migrating just below VP3 and VP4. An immune precipitated polypeptide is seen migrating slightly faster than VP5 (see also fig.4) and this could be VP5*.

A quantitative estimate of the efficiency of precipitation of each polypeptide was obtained by cutting out bands from gel tracks containing a) infected cells and b) immune precipitates, then solubilising the gel slices and counting the radioactivity in a scintillation counter (table 1). The gel seen in fig.10 was used. Since equal TCA-precipitable c.p.m. were loaded onto each gel track and the immune precipitates contained relatively less radiolabelled host cell proteins than the infected cells, each immune precipitated polypeptide should have more c.p.m. than the polypeptide in the original sample. If the immune precipitated polypeptide had fewer c.p.m. than the original polypeptide then it was considered not to be precipitated. By this criterion VP1, VP5 and VP10/10c were not precipitated, while VP2, VP3 and 4, VP4.2, VP6, VP7 and VP8 were efficiently

Table 1

Quantitation of immune precipitation of calf rotavirus polypeptides

by G203*

Protein (VP)	Inf. Cells	I.P.	Ratio ^{IP} /Inf.	Precipitated
1	3,600	2,600	0.72	-
2	10,400	81,600	7.85	+
3, 4	14,200	76,100	5.36	+
5	5,800	4,400	0.76	-
6	33,100	373,000	10.96	+
7	12,500	37,600	3.01	+
8, 9	34,300	116,000	3.38	+
10/10c	18,800	5,900	0.33	-

*Each radioactive band was excised from the gel, cut into small pieces and incubated in H₂O₂ at 80°C for 3h until dissolved. Triton/toluene/PP0 scintillation fluid was then added and the radioactivity in each slice was measured in a scintillation counter.

5.4 (Cont'd.)

precipitated. However VP10/10c were sometimes seen in immune precipitates.

5.5 Detection of rotavirus glycoproteins by immune precipitation using Con A-Sepharose.

Concanavalin A is a lectin protein which has highly specific sugar binding properties and can precipitate polysaccharides and glycoproteins (Nathan and Lis, 1972). It reacts specifically with α -D-mannose, α -D-glucose and related sugars. Con A covalently bound to Sepharose beads has been usefully exploited in affinity chromatography of many glycoproteins and I attempted to use this method to identify rotavirus glycoproteins in infected cell extracts. However, two main problems hampered results. Firstly, the denaturing conditions used for the affinity chromatography were too mild to completely dissociate intracellular protein complexes so eluates from the column contained a number of polypeptides that had been non-specifically bound. The second problem was that the glycoproteins bound so strongly to the column that only a small proportion of them could be eluted by competition with α -methyl-D-mannoside. An immune precipitation procedure was developed which avoided both these problems and was based on the use of Con A-Sepharose to bind immune complexes containing glycoproteins but not immune complexes of non-glycosylated proteins (Helmer and Klein, 1978). Immunoglobulin contains carbohydrate including mannose and

5.5 (Cont'd.)

N-acetyl-glucosamine (Kornfield *et al.*, 1971) but, at least when it is in the form of immune complexes, these carbohydrate chains must not be exposed as they do not bind to Con A (Heimer and Klein, 1978). Fig.7 compares proteins immune precipitated with protein A-Sepharose, which binds to the Fc region of immunoglobulin and Con A-Sepharose which binds sugar residues. Protein A-Sepharose precipitated immune complexes containing the normal rotavirus polypeptides seen in immune precipitates, while Con A-Sepharose specifically precipitated immune complexes of only two polypeptides - VP7 and VP10. Fig.7 also shows that the polypeptides precipitated by Con A without antibody contain several non-viral polypeptides, which are likely to host glycoproteins, while those precipitated by Con A-Sepharose in the presence of antibody contain only rotavirus glycoproteins. The fact that glycoprotein-antibody complexes were more easily precipitated than glycoproteins by Con A-Sepharose was possibly due to the fact that the antigens were present as large complexes and may have undergone conformational changes allowing better presentation of the sugar residues. This combination of antibody and Con A-Sepharose was therefore useful for reducing the host glycoprotein or non-specific background in the immune precipitates of rotavirus glycoproteins. Protein A-Sepharose without antibody also binds to a number of non-viral polypeptides. This is probably non-specific binding as it is not found when immune complexes are precipitated by Protein A-Sepharose.

Fig.7 Immune precipitation with Con A-Sepharose for identification of glycoproteins

Cells infected with 5 p.f.u./cell rotavirus and labelled with ^{35}S -methionine at 6-6½h p.i. were immune precipitated, using Protein A-Sepharose or Con A-Sepharose. Un = uninfected cells, In = infected cells. IP = immune precipitates of infected cells using α -calf rotavirus (CI) serum and protein A-Sepharose CP = immune precipitates of infected cells using CI serum and Con A-Sepharose IPc = immune precipitates of infected cells using no serum and protein A-Sepharose, CPc = immune precipitates of infected cells using no serum and Con A-Sepharose, PI = infected cells immune precipitated with preimmune serum and protein A-Sepharose. Numbers on the left of the photograph refer to rotavirus proteins seen in infected cells.

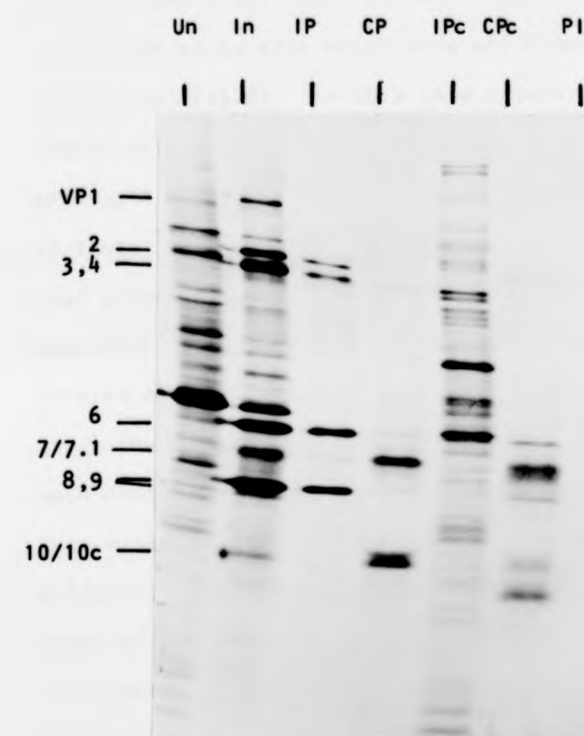
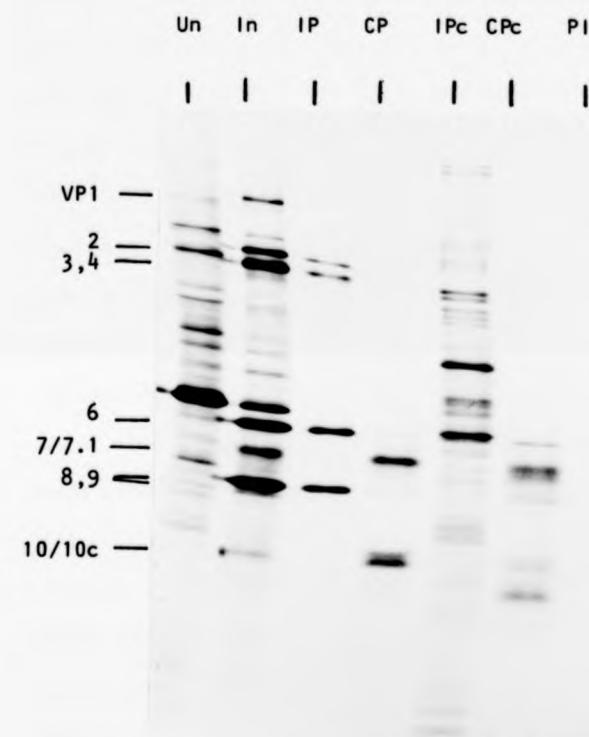


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5.6 Identification of polypeptides immune precipitated from infected cells with both homologous and heterologous antisera.

Calf rotavirus polypeptides were immune precipitated from infected cells with hyperimmune antisera against calf, human and pig rotaviruses, convalescent antisera against calf, human, pig and lamb rotaviruses and with newborn calf, foetal calf, rabbit and horse sera which were all known to contain rotavirus antibodies (fig.8). In this case heterologous antisera precipitated all the rotavirus polypeptides which had reacted with anti-calf rotavirus serum. The antisera used are all described in table 1, chapter 2. This preliminary result indicated that either 1) the rotavirus polypeptides were complexed together in infected cells, possibly as partly assembled viruses or simply as aggregates with affinity for each other, or 2) that no polypeptide is completely type-specific but each contains both type-specific and group-specific antigenic determinants on the same molecule. Molecules with several antigenic 'domains' have been identified by the use of a panel of monoclonal antibodies all against one protein where individual antibodies have different reactions with reoviruses (Burstin et al., 1982; see 1.3.2.6) and influenza virus (Gerhard et al., 1981). The existence of proteins complexed together as immature particles has also been described in reovirus (Joklik, 1981) and rotavirus (McNulty, 1979). This latter possibility was investigated first by attempting to dissociate protein aggregates before immune precipitation.

Fig.8 Immune precipitation with calf rotavirus
Infected cells with homologous and hetero-
logous antisera.

Cells, infected and labelled as before, were immune precipitated with a series of sera and the precipitates were analysed by PAGE. The antisera used are some of those described in table ^{1ch} 2. Virus proteins are labelled on the right of the photograph.

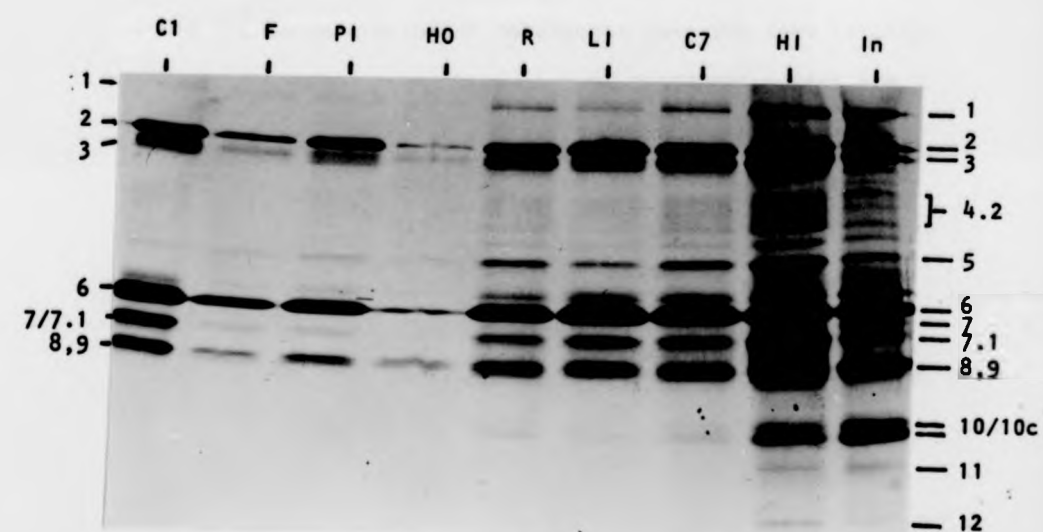
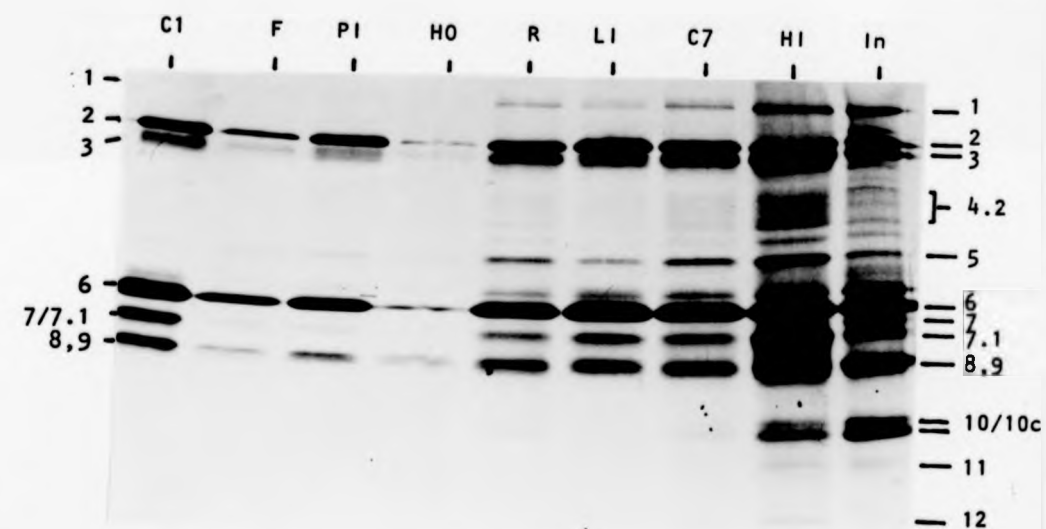


Fig.8 Immune precipitation with calf rotavirus
infected cells with homologous and hetero-
logous antisera.

Cells, infected and labelled as before, were immune precipitated with a series of sera and the precipitates were analysed by PAGE. The antisera used are some of those described in table^{1,2}. Virus proteins are labelled on the right of the photograph.



5.6 (Cont'd.)

Gentle sonication of cell extracts after 20 minutes at 37°C in buffer with 10 times (10X) the normal detergent concentration (10% SDS, 10% sodium deoxycholate, 5% Triton X-100) or with a 5X concentration of detergents was tried. The cell extracts were then diluted to a normal detergent concentration and immune precipitated as before. Pre-treatment of the cells with both a 5X and 10X concentration of detergents gave the same results: those for cells pretreated with 5X detergent buffer are shown in fig.9. The use of higher concentrations of detergents allowed identification of some type-specific differences presumably due to the detergent disaggregating the antigens. Both types of anti-calf rotavirus serum (C1 and C3) precipitated all the usual polypeptides except VP10, but anti-porcine rotavirus serum (P1) failed to precipitate VP7.1 and VP4.2 and precipitated VP3 much less efficiently than the other antisera. Hyperimmune serum against human rotavirus (H1) precipitated VP3 and VP7.1 but not VP4.2 and the non-structural polypeptide VP8. The precipitates in the region of VP4.2 appear as a diffuse area with at least four polypeptide bands. It should be noted that H1 antiserum has been raised against both subgroups of human rotavirus, one of which cross-reacts with calf rotavirus by ELISA. The efficiency of precipitation of each polypeptide by the antisera was quantitated by excising

Fig.9

Infected cells labelled with ^{35}S -methionine were treated with 5x detergent buffer (2.5% SDS, 5% sodium deoxycholate, 5% NP_{40} in 100 mM KCl, 5 mM MgCl_2 , 100 mM Tris pH8.2) at 37°C for 20 min, diluted to 1x detergent buffer and immune precipitated with calf, pig and human rotavirus antisera. The antisera are described in table 2. In = infected cells, Un = uninfected cells, IP are immune precipitates, Sup = material not precipitated by each of the antisera. Virus proteins are labelled on the left hand side of the photograph.

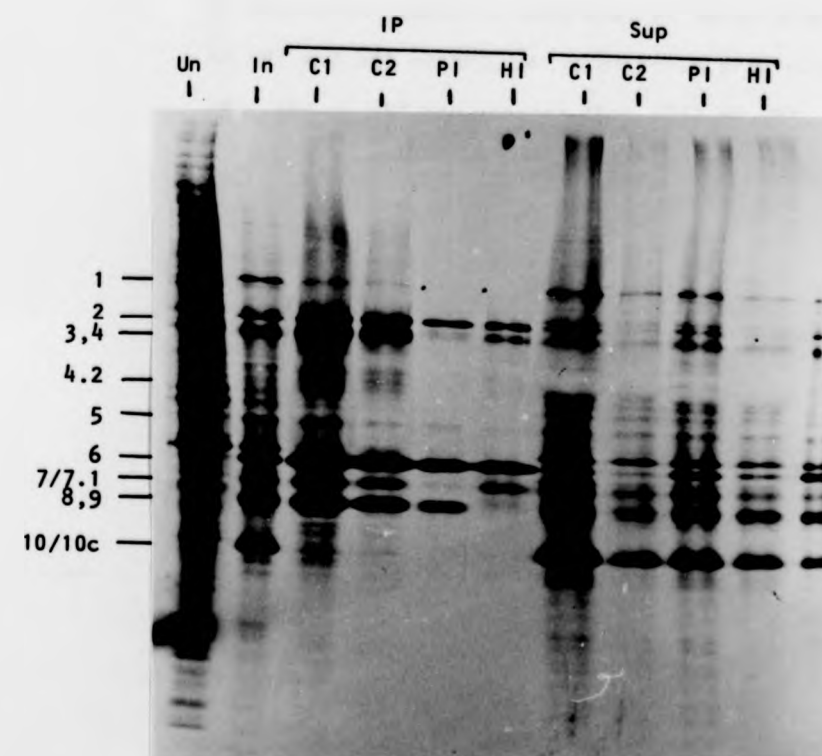
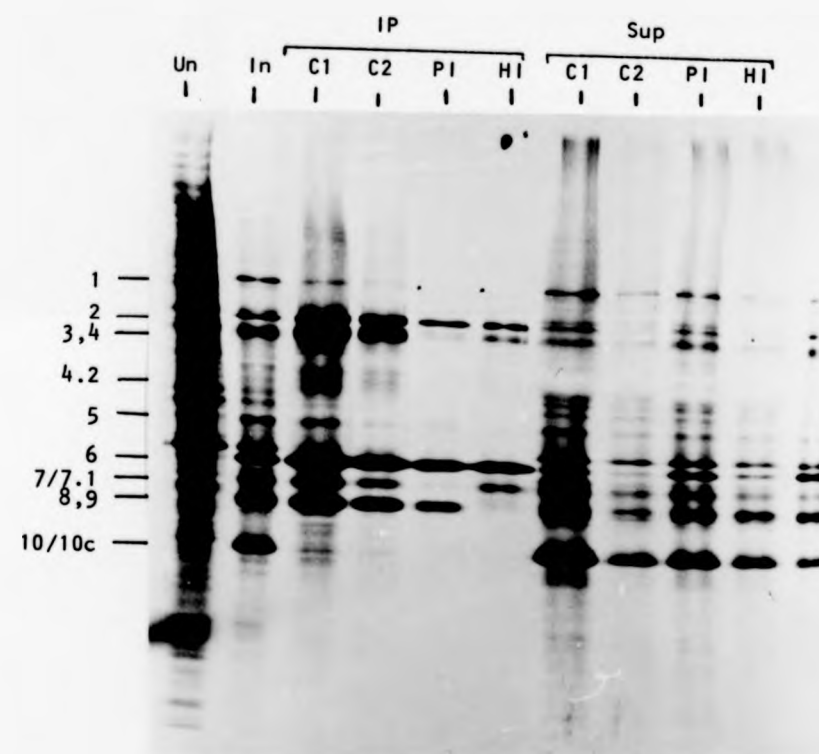


Fig.9

Infected cells labelled with ^{35}S -methionine were treated with 5x detergent buffer (2.5% SDS, 5% sodium deoxycholate, 5% NP_{40} in 100 mM KCl, 5 mM MgCl_2 , 100 mM Tris pH8.2) at 37°C for 20 min, diluted to 1x detergent buffer and immune precipitated with calf, pig and human rotavirus antisera. The antisera are described in table 2. In = infected cells, Un = uninfected cells, IP are immune precipitates, Sup = material not precipitated by each of the antisera. Virus proteins are labelled on the left hand side of the photograph.



5.6 (Cont'd.)

each band and determining the radioactivity in them.

The quantitative results comparing the amount precipitated by C2, P1 and H1 relative to the homologous C1 antiserum (table 3) show that P1 serum precipitates 23% of VP7 and 25% of VP3, while H1 serum precipitates 65% of VP3, 26% of VP8 and greater than 100% of VP7. Comparison of the precipitates of P1 and C1 using 1X detergents (fig.8) and 5X detergents (fig.9) show that the differential precipitation of VP7 and VP3,4 is less marked in lower detergent concentration. In fig.8, P1 does precipitate VP7 but to a much lesser degree than C1 and P1 also appears to precipitate less VP3, VP4 and VP4.2 than C1, but this difference is more obvious in fig.9. These polypeptides are possibly aggregated with others in infected cells and co-precipitate with them except in the presence of high concentrations of detergent. Heterologous antisera H1 and L1 appear to precipitate all rotavirus polypeptides in fig.8, while H1 does not precipitate VP8 and only some of VP3, VP4 and VP4.2 polypeptides in fig.9. Antisera F, Ho and R are not specific antisera.

These differences in precipitation of individual polypeptides by the antisera could result from genuine differences in the type specificity of rotavirus antigens

Table 3

Relative proportions of calf rotavirus polypeptides immune precipitated by α -calf, α -porcine and α -human rotavirus antisera.

Protein (VP)	c.p.m. precipitated by: Antisera				†% precipitated by:		
	C1	C4*	P1*	H1*	C4*	P1*	H1*
					relative to C1		
1	2,602	195	-	-			
2	81,620	94,977	68,170	66,890	116.4	83.5	81.9
3,4	76,063	74,085	19,660	48,906	97.4	25.6	64.3
5	4,410	2,825	2,936	5,565	64.1	66.4	126.2
6	373,038	370,993	313,460	346,330	88.7	84.03	92.8
7	37,559	28,182	8,770	53,470	75.0	23.4	142.4
8,9	116,056	117,396	99,370	29,770	101.2	85.6	25.7
10/10c	5,910	390	320	7,040	6.6	5.4	119.1

*The c.p.m. were adjusted to standardise the counts in each sample - the c.p.m. precipitated by C4 were all multiplied by 3 and those in P1 and H1 were multiplied by 10.

†The control samples were proteins precipitated by G203 (C1).

For descriptions of all the antisera used in this chapter, see Table 2.

5.6 (Cont'd.)

or merely reflect differences in the antibody content of individual antisera. In order to distinguish between these two, two further experiments were done. One was to precipitate calf rotavirus polypeptides with as many calf antisera as were available in order to demonstrate uniformity of precipitate patterns by them all, and the other was to infect cells with porcine rotavirus and immune precipitate the polypeptides with different antisera.

Fig.10 shows proteins of cells infected with calf rotavirus precipitated by several antisera against calf rotavirus as well as with antisera against human, porcine and lamb rotaviruses, after pretreatment of the cells with 5X detergent buffer. In this case, the precipitation of VP7 by C1 was less efficient than previously noted in fig.8 and fig.9. VP6 was strongly precipitated by all antisera. VP8 was strongly precipitated by all antisera except the hyperimmune antisera raised against human rotavirus. VP2 was also precipitated by all antisera, although less strongly than VP6. This experiment distinguishes between VP3 and VP4. It has already been pointed out (chapter 4) that the structural polypeptide VP3 is the faster migrating of these two. The slower migrating VP4 is precipitated by all antisera while VP3 is only precipitated by H1 and four of the six calf

Fig.10 Immune precipitation with homologous and heterologous antisera

Cells infected with calf rotavirus were immune precipitated with a range of homologous and heterologous antisera as in fig.9. The antisera are described in table 1, ch2.

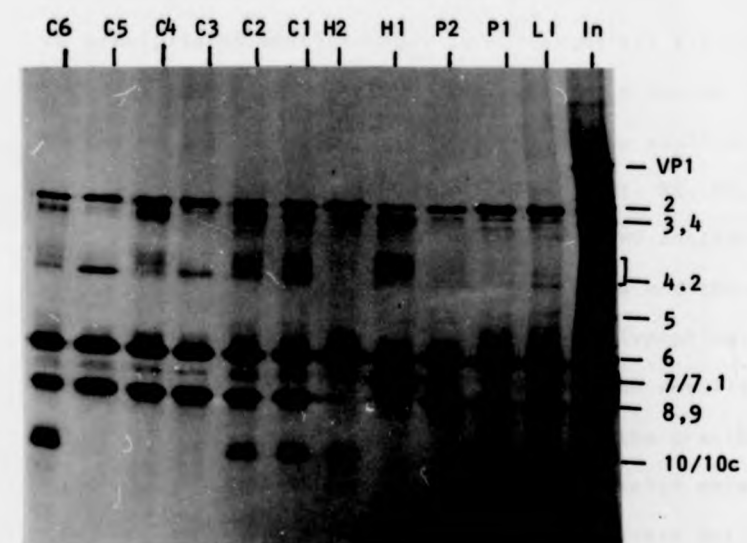
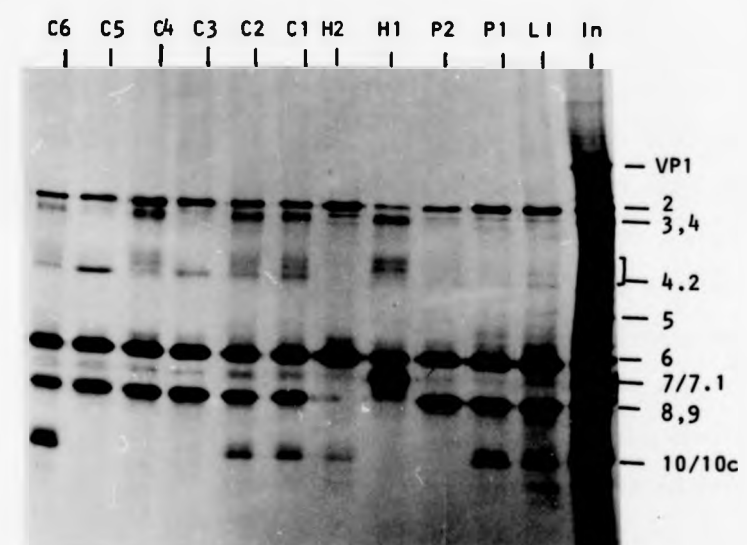


Fig.10 Immune precipitation with homologous and
heterologous antisera

Cells infected with calf rotavirus were
immune precipitated with a range of
homologous and heterologous antisera as
in fig.9. The antisera are described in
table 2, ch2.



5.6 (Cont'd.)

antisera. VP3 is not precipitated strongly by C5. The related polypeptide VP4.2 is precipitated by all calf antisera and by H1. Interestingly, at least three bands at VP4.2 are identifiable in the precipitates of H1 and four of the calf antisera, and only one band is seen in the precipitates of the other two calf antisera. VP7 is precipitated most strongly by H1 serum; all six calf antisera precipitated VP7 weakly, but precipitation of VP7 by H2, P1, P2 and L1 antisera was barely visible. VP10/10c was precipitated as two bands by P1, P2, H2, C1, C2 and C6. This experiment shows that (1) H2 antiserum differs from the other heterologous antisera and therefore its type-specificity is in doubt and (2) polypeptides VP3, VP4.2 and VP7 are type specific, although the results for VP7 shown in fig.10 are less clear than in the previous experiment (fig.9) as VP7 is poorly precipitated except by H1. VP10 is not precipitated by all antisera but is precipitated by both homologous and heterologous antisera.

5.7 Immune precipitation of cells infected with porcine rotavirus (OSU).

The other method of determining whether differences in precipitation of individual proteins by heterologous antisera did reflect the fact that there were type specific antigens or whether it simply reflected

5.7 (Cont'd.)

variation in individual antisera was to immune precipitate polypeptides from a different rotavirus serotype with all the antisera. Attempts to get sufficient expression of rotavirus polypeptides in infected cells from a number of fresh porcine rotavirus isolates not adapted to tissue culture were unsuccessful. A Canadian porcine rotavirus isolate which had been adapted to tissue culture (OSU strain), was kindly provided by Dr. Bohl, and polypeptides produced in infected cells by this rotavirus were immune precipitated with a range of antisera (fig.11). However, the experiment was largely inconclusive because (1) immune precipitation of many polypeptides, i.e. VP7, VP8 and VP9 was very weak in all cases and differences could not really be detected and (2) there was no evidence that OSU belonged to the same serotype as either of the two British porcine isolates used to raise our porcine antisera so all the antisera used in this experiment could have been heterologous. The OSU rotavirus did not form plaques so a plaque reduction assay with the antisera could not be carried out. Because of the frequent problem of variable precipitation of some of the antigens (namely the glycoproteins VP7 and VP10) by homologous antisera, it was felt that the experiments described in the next two chapters (characterisation of antigens using monospecific antisera) would yield more information than this approach, so no further experiments of this nature were carried out.

Fig.11 Immune precipitation of cells infected with porcine rotavirus OSU

Cells were infected with porcine rotavirus (OSU) as described for calf rotavirus, pretreated with 5x detergent buffer and immune precipitated. Unin = uninfected cells, In = calf rotavirus infected cells, OSU In = cells infected with OSU, OSU-IP = OSU infected cells immune precipitated with antisera labelled. (The antisera are described in table 2). Virus polypeptides are labelled on the right hand side.

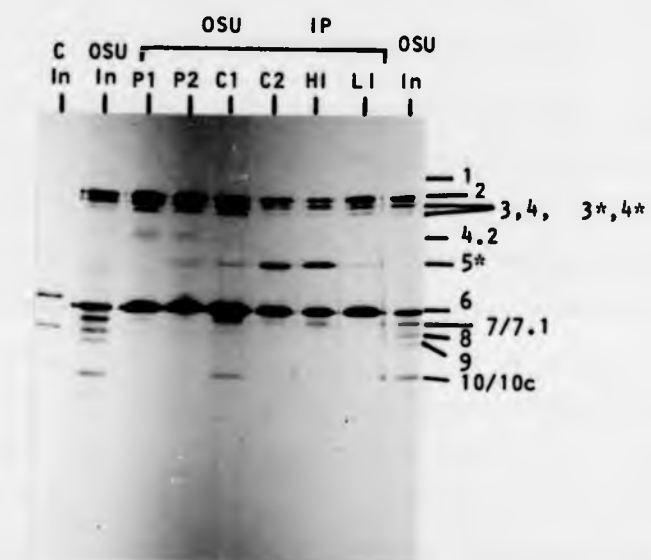
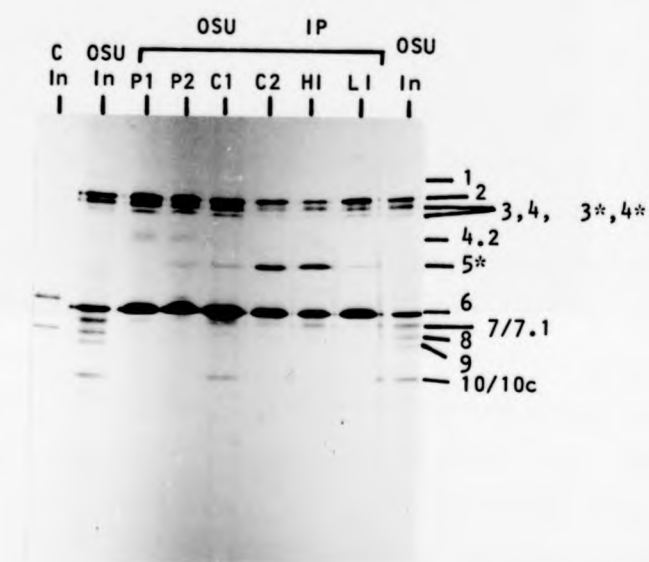


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Cells were infected with porcine rotavirus (OSU) as described for calf rotavirus, pretreated with 5x detergent buffer and immune precipitated. Unin = uninfected cells, In = calf rotavirus infected cells, OSU In = cells infected with OSU, OSU-IP = OSU infected cells immune precipitated with antisera labelled. (The antisera are described in table 2). Virus polypeptides are labelled on the right hand side.



DISCUSSION

This chapter describes the immune precipitation technique, optimisation of the conditions, verification of its specificity and a number of applications for its use. The specificity of the technique is confirmed in the next chapter where monospecific antisera are shown to precipitate only one polypeptide.

In the homologous calf rotavirus (C1) antiserum system a number of polypeptides seen in non-precipitated extracts of infected cells were immune precipitated (VP2, VP3, VP4, VP6, VP7/7.1, VP8 and VP10/10c). More importantly other polypeptides were precipitated and thus identified as putative rotavirus polypeptides: one migrating just below VP3 which may correspond to VP3*, 4*, one which migrates in the expected region of VP4.2 (this precipitate often appears as about three bands), one which migrates just below VP5 which could be VP5* and one migrating just below VP9 which migrates in the expected position of vpr7. These four polypeptides are precursors or cleavage products of rotavirus proteins. VP4.2, VP5* and VP3*, 4* are all found in virus particles and vpr7 is seen after in vitro translation. An added value of immune precipitation is shown in chapter 6 where monospecific antisera are used. The fact that VP4.2 was identifiable as three bands when there is only one in rotavirus particles could indicate more than one cleavage or other posttranslational step in its production or heterogeneity in the protein. However, we have no evidence that any or all of them are really VP4.2. Most of the rotavirus polypeptides were

precipitated by antisera - but the structural polypeptide VP1 and non-structural polypeptides VP5 and VP9 were not immune precipitated. VP11 and VP12 were weak bands if seen at all so the only conclusion we can make about them is that they were not enhanced by immune precipitation. VP12 is a precursor of VP10/10c but it is not enhanced as vpr7 is by immune precipitation, despite the obvious presence of antibody to VP10/10c. VP10/10c are modified from VP12 by glycosylation and cleavage steps, so it is possible that some of the antibody to VP10/10c is directed against the modified regions, but it is also possible that VP12 is converted to VP10/10c more efficiently conversion of vpr7 to VP7, leaving little to be immune precipitated.

The proportion of VP7/7.1 and VP10/10c which become immune precipitated by even the same antiserum varied from experiment to experiment. Precipitation of these polypeptides was often poor (although not always - see fig.9) when the cells were pretreated with high concentrations of detergent. In other virus systems including reovirus, herpes, respiratory syncytial virus and foot and mouth disease virus (Gaillard and Joklik, 1980; Yeo et al., 1981; Bernstein and Hruska, 1981; and Harris et al., 1981) immune precipitation of the type-specific polypeptide or the glycoproteins was less efficient than that of the other polypeptides. This could be the result of denaturation of the protein but later experiments (described in chapter 6) showed that omission of SDS and deoxycholate (DOC) did not improve precipitation of VP10 by α VP10. Possibly the

use of detergent buffer containing SDS and DDC without NP₄₀ may have worked as it has been observed that the presence of non-ionic detergents, which can solubilise membrane proteins by forming micelles around the hydrophobic regions of the proteins, often greatly reduce the binding of glycoproteins to Concanavilin A (Helenius and Simons, 1975; Gombos, 1976). Presumably they could also prevent glycoproteins or proteins with hydrophobic regions from binding to antibody if these hydrophobic regions were the antigenic sites. Ionic detergents presumably form micelles with more hydrophilic determinants. Systematic reduction in the concentration of each or all of the detergents to below their critical micellar concentration (CMC) may improve precipitation of the glycoproteins but may also decrease specificity.

We also attempted to distinguish group-specific from type-specific antigens. Pretreatment of cells in high concentrations of detergents improved the specificity of immune precipitation and differences in the immune precipitates of homologous and heterologous antisera were detected. Interpretation was difficult because of the variability in precipitation of the glycoproteins referred to above. However, we were able to determine that VP2 and VP6 were group-specific antigens since they were precipitated by all antisera in cells infected with OSU-porcine rotavirus or calf rotavirus. VP8 was also probably group-specific although, as a non-structural polypeptide, it was not precipitated by antisera against virus particles (H1 and H2), and was weakly

precipitated in cells infected with OSU. These polypeptides may have both group-specific and type-specific antigenic determinants and be precipitated by antibodies against the group-specific regions. The group/type specificity of VP1, VP4 and VP9 was not easy to determine as VP1 was not usually precipitated and VP4 and VP9 were not usually resolved from VP3 and VP8 respectively. VP3, VP4.2 and VP7/7.1 behaved as type-specific polypeptides by immune-precipitation from calf rotavirus-infected cells, but precipitation of VP7/7.1 in fig.10 was poor. VP3 and VP4.2 were type-specific as they were only precipitated with homologous antisera and the non-specific H1. An interesting observation was that homologous antisera either precipitated VP3 strongly or VP4.2 strongly. VP3 and VP4.2 are related polypeptides so this precipitation presumably depended on which portion of the protein the antibodies were directed against. Four of the six calf antisera precipitated three bands in the region of VP4.2, which suggests that VP4.2 is a heterogeneous mixture of proteins. VP10 was precipitated randomly by some homologous and some heterologous antisera.

Results from OSU-infected cells indicated that VP4.2 was only precipitated by porcine antisera but results of other proteins were difficult to interpret as VP7/7.1 and VP8,9 were precipitated weakly in all cases and VP3 was obscured by a number of other closely migrating bands, probably including more VP3*,4* than seen in calf rotavirus-infected cells, as

the other cleavage product of VP2 (i.e. VP5*) is seen in great quantity.

The attempts to find group-specific and type-specific antigens thus presented more questions than they answered. One problem may have been that convalescent antisera do not give such serotype-specific reactions as hyperimmune sera, and few hyperimmune antisera were available. Type-specificity may have been easier to demonstrate using antisera adsorbed against heterologous rotavirus to remove antibodies against group-specific determinants, but this would not remove antibody against internal antigenic sites and, as precipitation of the glycoproteins was not reproducible, the type-specificity of these glycoproteins would not be demonstrable in any case. The next chapter on monospecific antisera provides some indication that VP10 is type-specific in immunofluorescence and shows that VP7.2 is involved in neutralization, seen by Bastardo et al. (1981) to be type-specific. VP4.2 is also likely to be type-specific as it is an outer shell protein formed by trypsin action and necessary for infection of cells, and VP3 is its precursor. Type specificity may be easier to demonstrate by immunoprecipitation with monospecific or monoclonal antisera, but the monospecific antisera we have raised were against denatured proteins (so type-specific determinants may have been destroyed) and they immune precipitated proteins from OSU-rotavirus as well as from calf rotavirus (chapter 6).

Glycoprotein antigens were also identified by an adaptation of the immune precipitation technique which is specific and therefore a useful method for future use. This chapter therefore served as an introduction to the characterisation of antigens described in chapters 6 and 7 as we were able to differentiate antigens which were group specific, not entirely group-specific and identify glycosylated antigens.

CHAPTER 6

The purpose of this chapter is to provide a detailed description of the various types of data that can be collected and analyzed. The chapter is divided into two main sections: the first section describes the various types of data that can be collected, and the second section describes the various types of data that can be analyzed.

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CHAPTER 6

Preparation of monospecific polyclonal antisera against individual rotavirus antigens, and investigation of their reaction with rotavirus polypeptides.

Introduction: This section describes the preparation of monospecific antisera against each of the rotavirus structural polypeptides and their application in the further antigenic characterisation of rotavirus. This method has been useful in many virus systems - monospecific antisera have been raised against viral polypeptides isolated under mild conditions in the case of Influenza virus and others (Eckert, 1966; Wrigley et al., 1977; Rott, 1964; Hayes et al., 1981), but in many cases it has been necessary to use denatured and reduced polypeptides excised from polyacrylamide gels (Powell et al., 1974; Lane and Robbins, 1978; Hayes et al., 1981) and these polypeptides have proved to be not only antigenic but to retain some of their native configuration so that the antisera against them had the expected type specificity and neutralizing properties. This latter approach was used for the preparation of rotavirus monospecific antisera described in this chapter. Obviously, the success of this approach is dependent on stability of the antigenic configuration of these polypeptides to denaturation and reduction, as well as on the monospecificity of the antisera raised, and these factors will be discussed. The antisera were useful in determining the relationship of rotavirus antigens to each other and in

Introduction (Cont'd.)

Identification of glycoproteins and their precursors, as well as in identifying individual biological functions of the rotavirus antigens, particularly those which were concerned with neutralization, by comparison of the effects of the monospecific antisera in their reactions with virus. The ability to bind to, neutralize and aggregate virus particles was investigated as well as identification of the cellular location of individual rotavirus antigens in infected cells by immunofluorescence.

This chapter describes the preparation and screening of the monospecific antisera and their reactions with rotavirus polypeptides in immune precipitation and immunofluorescence, and chapter 7 describes the reaction of monospecific antisera with virus particles. The definition of a monospecific antiserum is that it is raised against and only reacts with a single protein (and its precursors or modified products).

6.1 Preparation of monospecific polyclonal antisera against individual rotavirus polypeptides.

6.1.1 Maintenance of rotavirus-free guinea pigs.

A major initial problem in raising monospecific antisera was the acquisition and maintenance of animals without rotavirus antibody. A preliminary screen of rabbit serum from some young rabbits in our animal house showed that the serum precipitated rotavirus polypeptides. Guinea pigs were chosen for these experiments following a report by Zissis and Lambert (1980) that 90% of guinea pigs tested were free of rotavirus antibody whereas rabbits frequently had rotavirus antibodies under normal management conditions. In an initial experiment, a group of uninoculated animals were kept in the isolation conditions described in Methods (Chapter 2) for six weeks. The sera from these guinea pigs developed no rotavirus antibody over the test period as shown by immunoprecipitation (fig.1). The test animals used in subsequent experiments also remained free of antibody to rotaviruses for the 12 month duration of the experiment.

6.1.2 Preparation of antigen.

Individual rotavirus polypeptides were obtained by PAGE of rotavirus purified from tissue culture. For each injection, rotavirus was grown in and purified from 30-40 roller bottles (approximately 1.5×10^7 cells), which provided on average 1 mg of purified double shelled virus. A small amount of ^{35}S -methionine labelled

Fig.1 Immune precipitation of rotavirus polypeptides with pre-immune antisera.

Infected cells (In) and uninfected cells (Un) were immune precipitated (IP) with C1 serum and preimmune sera from guinea pigs labelled G1, G2 and G3. Rotavirus polypeptides seen in infected cells are labelled on the left hand side.

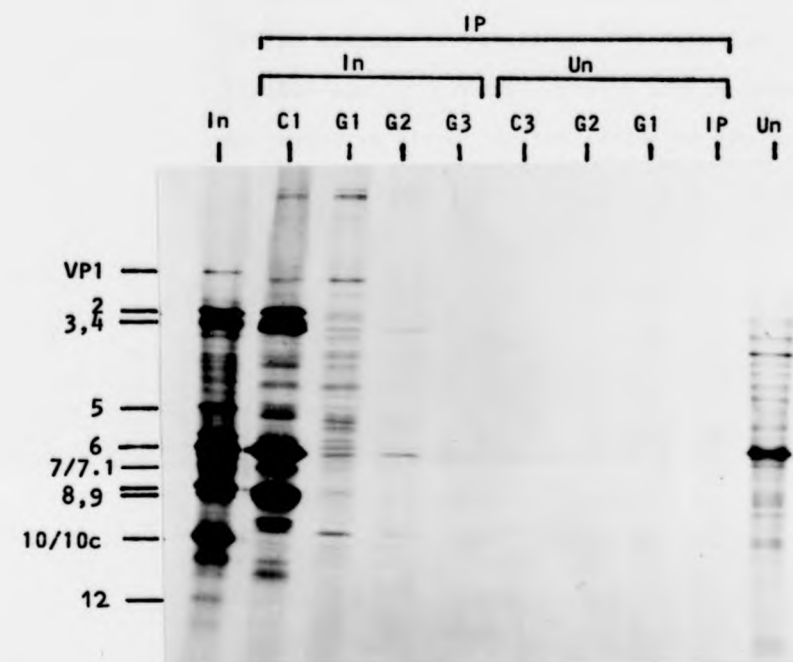
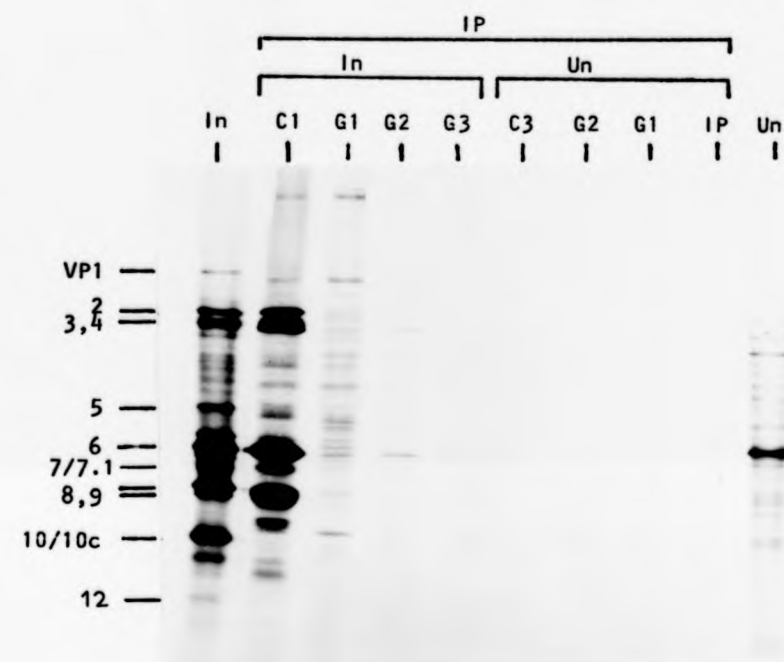


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6.1.2 (Cont'd).

rotavirus (50,000 cpm ^{35}S -labelled rotavirus mixed with 200 μg unlabelled virus) or ^{125}I labelled rotavirus (20,000 cpm ^{125}I rotavirus with 200 μg unlabelled virus) was added to allow identification of the virus bands.

Fig.2 is a PAGE pattern of rotavirus structural polypeptides labelled with ^{35}S -methionine or ^{125}I , as described in chapter 4. The structural polypeptides VP1, VP2, VP4.2, VP6, VP7.1, VP7.2 and VP10 were excised individually as immunogen. VP3* and VP4* were excised together as they were difficult to separate and also injected together.

6.1.3 Development of antibody response in guinea pigs

The course of antibody production by immunised guinea pigs was monitored by both immune precipitation and immunofluorescence. One animal was injected with acrylamide containing no rotavirus protein to serve as a control. Several test bleeds were taken after each injection to identify the time of peak antibody production. Antibody production was estimated to be highest 12 days after injection by testing the efficiency of the serum in immune precipitation. The results of screening guinea pig sera after each injection are shown in table 1, and the pattern of development of an antibody response for each polypeptide can be seen from it. The variation in rate of development of specific antibody against each

Fig.2 PAGE of single shelled (ss) and double shelled (ds) rotavirus particles radiolabelled with [^{35}S]-methionine or ^{125}I . Uninfected (U) BSC-1 cells and cells infected (I) at 10 p.f.u./cell and labelled with [^{35}S]-methionine from 6 to 6.5h post infection were also included. Numbering of the polypeptides is explained in chapter 4. The scheme on the right refers to virus polypeptides present in infected cells and that on the left to polypeptides (arrowed) which are seen in virus particles.

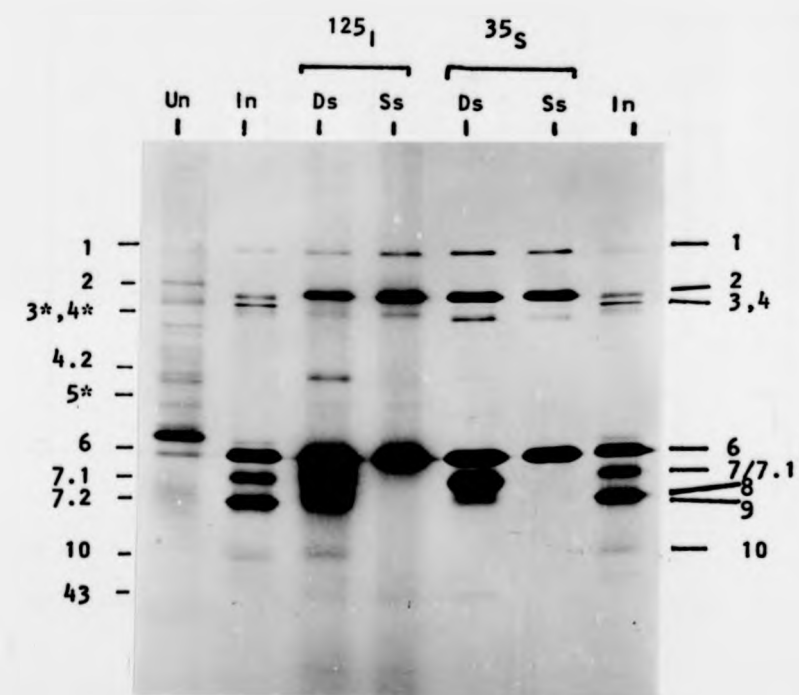


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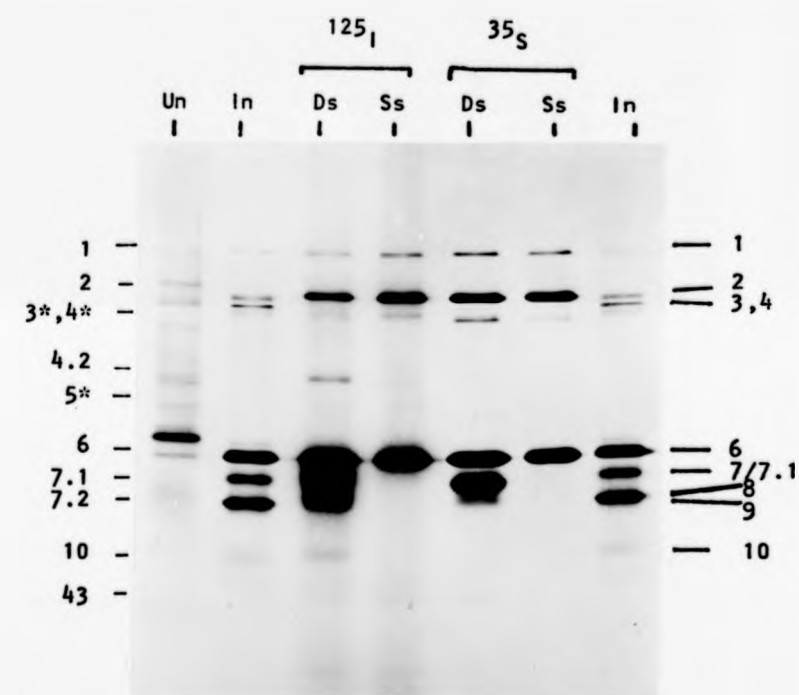


TABLE 1

Development of guinea pig antibodies to proteins
purified from rotavirus particles

PROTEIN VP	INJECTION NO.													
	2		3		4		5		6		7		8	
	IP*	IF	IP	IF	IP	IF	IP	IF	IP	IF	IP	IF	IP	IF
1	†	-	-	-	-	-	-	-	-	+	-	+	-	+
2	+	+	++	++	++	++	++	++	++	++				
3*,4*#	-	-	-	-	-	-	-	-	-	-	-	±	±	+
4.2	-	-	-	-	-	-	-	-	-	±	-	±	-	±
6	-	-	+	+	+++	++	+++	+++						
7.1	-	-	+	++	+++	+++	+++	+++						
7.2	-	-	-	-	-	++	++	+++	+++	+++	+++	+++	+++	+++
10	-	-	-	-	-	-	-	++	-	+++	-	+++	-	+++

† The antibody status is graded from no response (-), significantly positive (+) to strongly positive (+++).

* IP = immune precipitation, IF = immunofluorescence.

The antibody response to VP3*,4* was shown by immune precipitation to be against VP2; all others were specific for the immunogen used.

6.1.3 (Cont'd.)

polypeptide was not simply related to the quantity of immunogen injected. While in general the most prominent polypeptides (VP2, VP6 and VP7.1) produced the earliest immune response, the relationship between quantity of immunogen injected and the immune response it elicited was complicated. We have estimated the percentage of each polypeptide in the virus particles from our own data and that provided by Novo and Esparza (1981), and from this we have estimated the quantity of each polypeptide injected (see table 2). Of inner shell proteins, an estimated 10-15 µg of VP1 was injected and a poor immune response was seen only after 6 injections. 100-200 µg and 400-600 µg of VP2 and VP6 were injected respectively but the immune response to VP2 was seen earlier than that to VP6, although VP6 eventually elicited a good immune response. Of the major outer shell proteins, 120-160 µg of VP7.1 and 50-80 µg of VP7.2 were injected. An immune response to VP7.1 was seen as quickly as that to VP6. The response to VP7.2 lagged behind by one injection. 6-10 µg of VP10, 4-8 µg of VP3* 4* and 10-15 µg of VP4.2 were injected, and of these, VP10 elicited an excellent immune response while that to the others was poor.

Final bleeds were taken when the antibody response reached a plateau or, in the case of VP1, VP3*, 4* and VP4.2 at the end of the experiment. For most polypeptides,

6.1.3 (Cont'd).

antiserum was collected after each of a number of injections but, as the amount of antibody in each serum sample varied, one sample was chosen of the sera against each polypeptide for use in subsequent experiments.

6.2 Characterisation of monospecific antisera by immune precipitation of infected cell extracts and purified virus.

Immune precipitation was used both to screen for development of an antibody response, and to test the specificity of the monospecific antisera. The screening results are shown in Table 1. Initially, immune precipitation followed by PAGE showed that the sera were not monospecific. The possibility that aggregates in infected cells were precipitated by antisera against any one of them was investigated by using more stringent denaturing conditions containing 2% SDS and 5% BME at 100°C for 2 min. Samples were denatured by this treatment before dilution in detergent buffer for immune precipitation. As can be seen in figs. 3 and 4, this pretreatment dramatically increased the specificity of immune precipitation. Before denaturation, α VP2 and α VP6 both precipitated VP2, VP3, VP6 and a polypeptide migrating below VP3 (fig.3). These polypeptides therefore

Fig.3 Cells infected with 5 p.f.u./cell calf rotavirus (In) were immune precipitated with foetal calf serum (C6), antiserum against calf rotavirus (C1) or with monospecific antisera α VP2 and α VP6. Two bleeds of each antiserum were used to immune precipitate non-denatured infected cell samples (Non-Denat.), and the results were compared to immune precipitates of denatured and reduced infected cell samples (Denat.), as explained in the text. The uninfected cell profile is shown on the right hand side. Virus proteins are labelled on the left hand side.

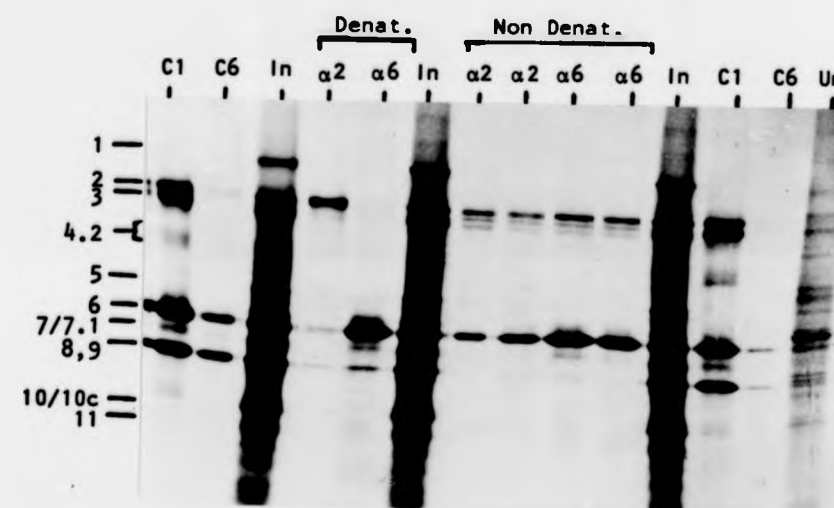


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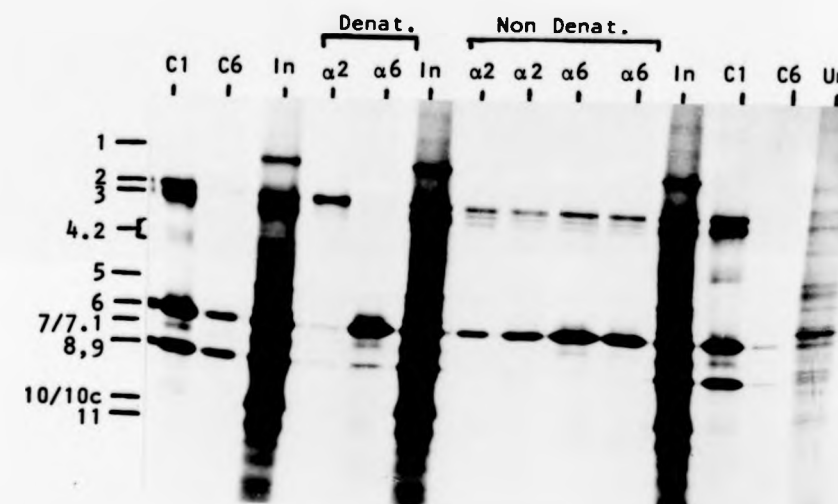


Fig.4 Cells infected with 5 p.f.u./cell calf rotavirus (In) were immune precipitated with antiserum against calf rotavirus (C1) or with monospecific antisera α VP7.1, α VP7.2 and α VP10. As in fig.3, immune precipitates of non-denatured infected cell samples (Non-Denat.) were compared to immune precipitates of denatured and reduced samples (Denat.). Two bleeds of α VP7.2 were compared using non-denatured cells. Virus proteins are labelled on the right hand side.

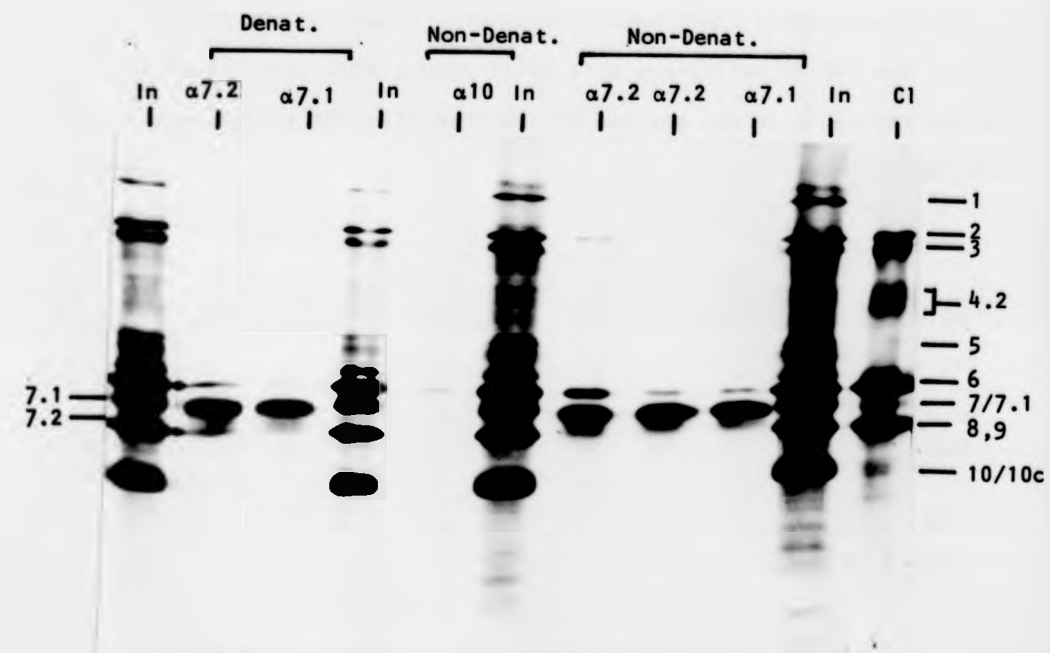
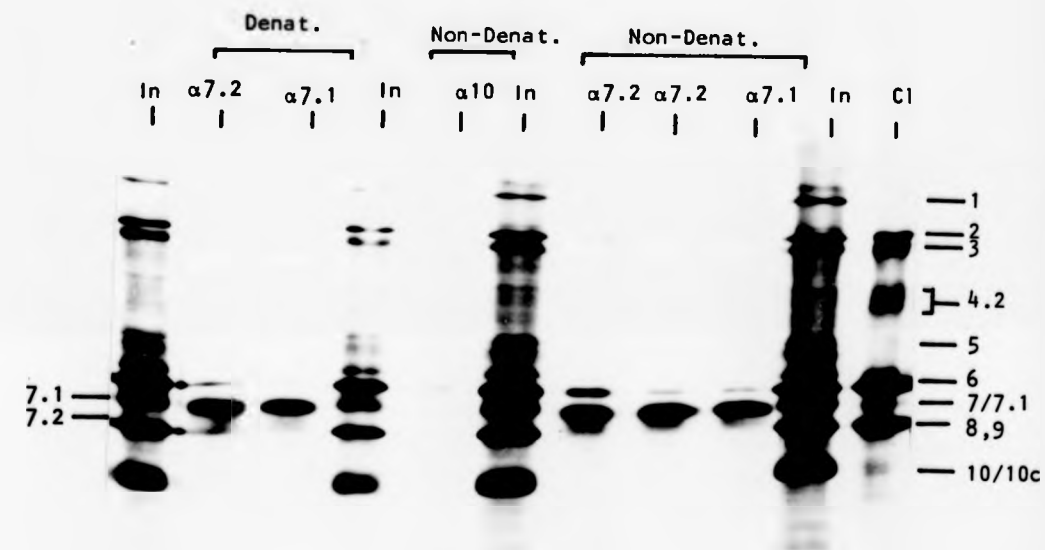


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6.2 (Cont'd).

form an aggregate resistant to the detergents in 'detergent' buffer. α VP7.1 and α VP7.2 both precipitated VP6 as well as VP7 (fig.4), so VP6 and VP7 also associate in infected cells. α VP10 did not react under either condition. Under denaturing conditions, α VP6 precipitated tiny quantities of VP7 and VP10 as well as the main band of VP6 but the amount of contamination was low and rarely observed so probably represented non-specific precipitation.

Under optimum immune precipitation conditions, shown in fig.5, α VP2 precipitated only VP2, α VP6 precipitated only VP6 and α VP3*,4* precipitated only VP2. VP3*,4* are found only in virus particles and not in infected cell extracts and they are now thought to be cleavage products of VP2 (Estes *et al.*, 1981) so these facts could explain this precipitation result. However, contamination of the immunogen with VP2 cannot be ruled out. α VP7.1 and α VP7.2 both precipitated the major polypeptide VP7 and two minor polypeptides migrating below VP7, with molecular weights similar to those expected of VP7.1 and VP7.2. In some cases (see fig.8) two major polypeptides were precipitated as well as one or two minor ones. This may reflect the amounts of VP7, VP7.1 and VP7.2 in cell extracts available for immune precipitation. This is difficult to determine because on PAGE VP7.2 may be masked by VP8 even when present in fairly large quantities. In the case of fig.8, it appears that the major precipitates are of VP7

Fig.5 PAGE of immune precipitation from [^{35}S] methionine-labelled cell extracts infected with 5 p.f.u./cell and pulse labelled from 6 to 6.5h post-infection. Tracks show the unprocessed infected cell extract (In) or extracts precipitated with the various antisera (α). Two minor polypeptides precipitated by $\alpha\text{VP7.1}$ and $\alpha\text{VP7.2}$ are arrowed. Numbers on the left and right refer to virus polypeptides of infected cells.

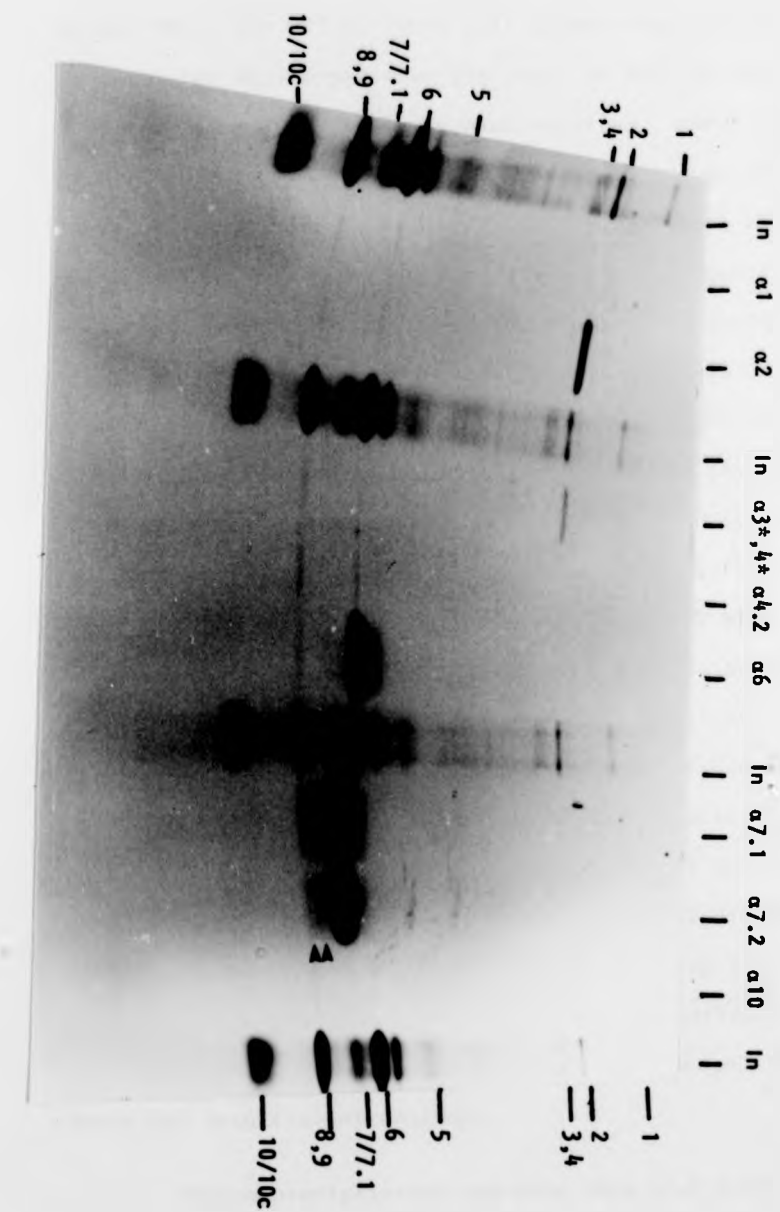
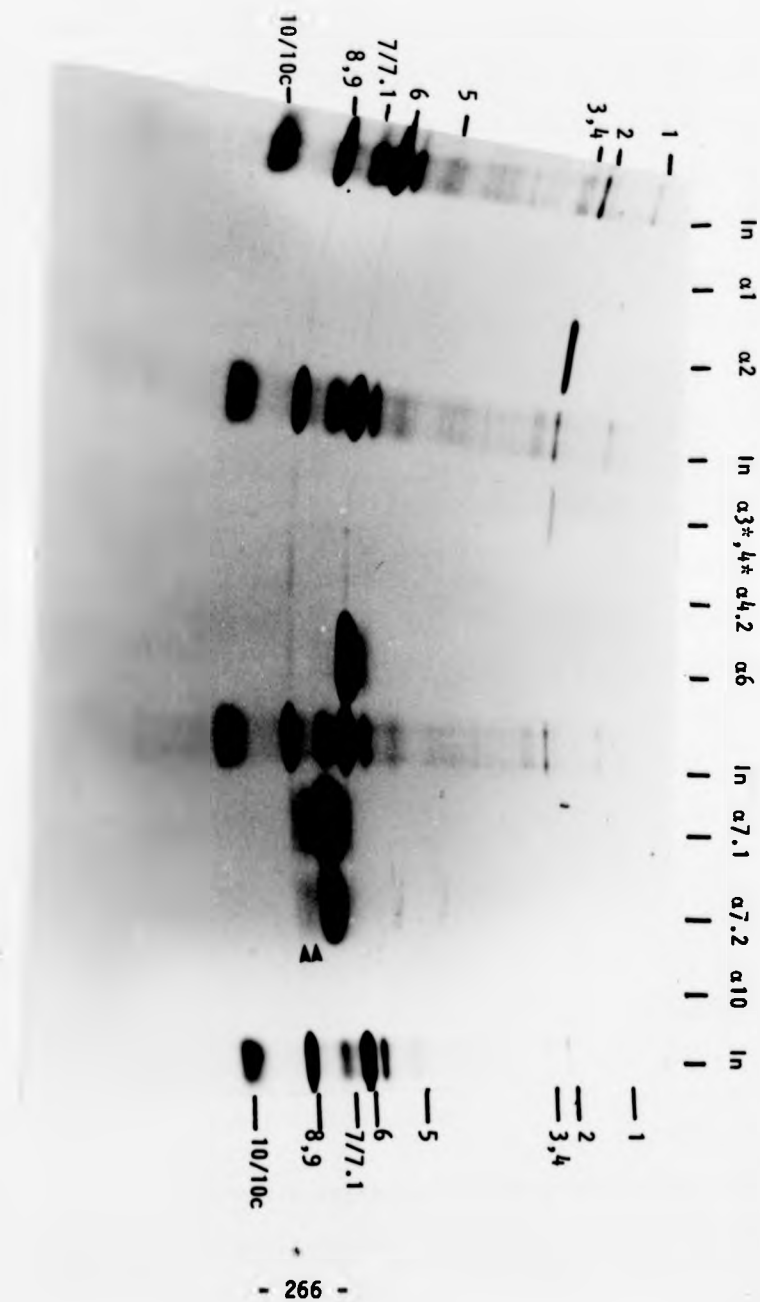


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6.2 (Cont'd).

and/or VP7.1 and VP7.2. This cell extract was pulse labelled for 1h (longer than the usual 30 min) so more cleavage steps than usual may have occurred. α VP10 never precipitated VP10 from infected cells, even though the antibody response of VP10 was judged to be high by bright, specific immunofluorescence. In fig.5, small amounts of VP2 were precipitated but this was an aberrant result as it was only observed on this occasion. The possibility that α VP10 bound only weakly to VP10 and that this binding was diminished by standard detergent buffer was investigated by attempting to immune precipitate with reduced quantities of detergent in the buffer. Antigen was prepared and immune complexes precipitated in buffer containing 0.5% NP40 as the only detergent, but VP10 was still not precipitated under these conditions. The fact that VP10 is precipitated very weakly by all polyspecific antisera has been discussed in chapter 5. This is not due to lack of immunogenicity of VP10, and later experiments show that it is not due to lack of antibody in α VP10 antiserum. α VP1 and α VP4.2 did not precipitate any protein from infected cells (fig.5), but as these two antisera also gave a very weak immunofluorescent response (fig.7), this was probably due to the fact that there was insufficient antibody.

Immune precipitation was also done with each of the monospecific antisera and ^{35}S -methionine labelled virus

6.2 (Cont'd.)

because (1) antisera that precipitated specific polypeptides in infected cells could behave differently with purified virus, e.g. by recognising further modifications of the polypeptide, (2) antisera against polypeptides present in purified virus but not easily demonstrated in infected cells (e.g. VP4.2) may precipitate these polypeptides from virus particles, (3) α VP7.1 and α VP7.2 might be distinguishable from one another by the precipitates they form from virus particles (i.e. if VP7.1 and/or VP7.2 have a different configuration in virus particles than in infected cells the antisera may react specifically with α VP7.1 or α VP7.2). However in all cases the immune precipitation patterns with these antisera were the same as with infected cell extracts (fig.6). The immune precipitation pattern from purified virus with α VP7.1 and α VP7.2 was simpler as they both precipitated VP7.1 and VP7.2 only. These polypeptides were precipitated by α VP7.1 and α VP7.2 to the same extent and both antisera precipitated VP7.2 weakly. This was probably due to the fact that VP7.2 is a minor polypeptide, (but the antigenicity of VP7.2 may be susceptible to detergent or other factors in the immune precipitation reaction). VP10 was not precipitated from purified virus, nor were VP3*,4*, VP4.2 or VP1.

Fig.6 PAGE of immune precipitation of polypeptides from rotavirus particles grown in the presence of ^{35}S -methionine. The virus particle profile (V) is shown on the left hand side and infected cells (In) are used as marker tracks. The immune precipitates of the various monospecific antisera (α) are labelled at the top. Numbers on the left refer to polypeptides from rotavirus particles and those on the right to virus polypeptides of infected cells.

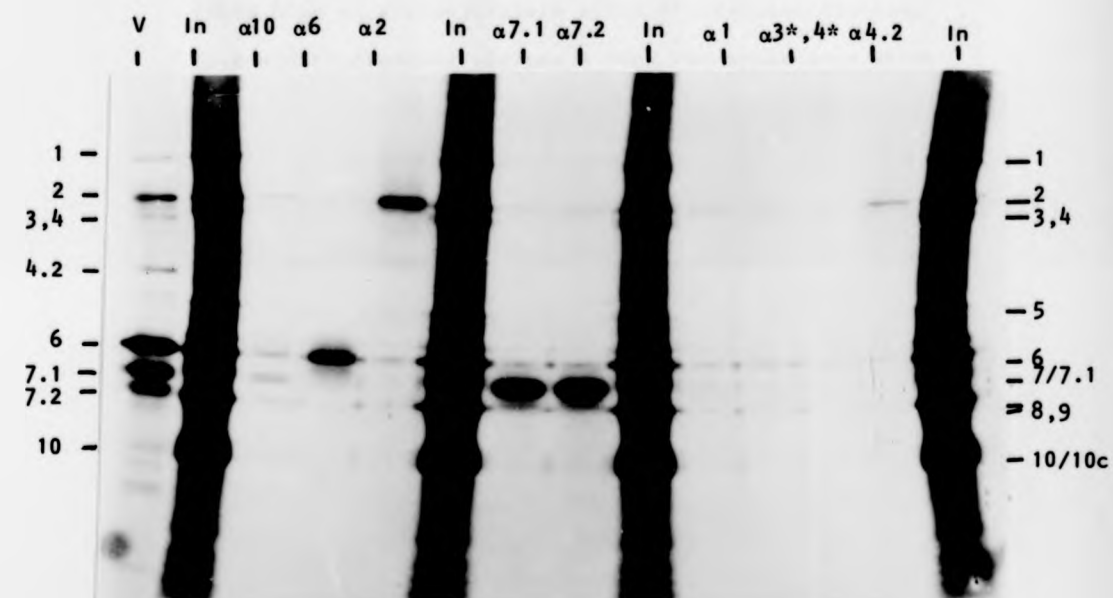
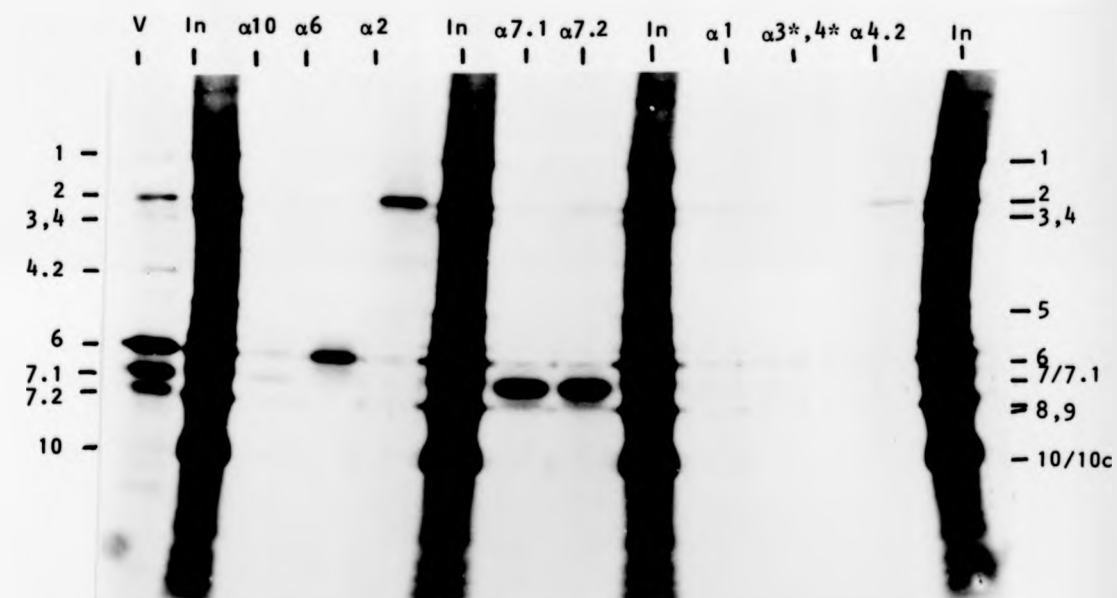


Fig.6 PAGE of immune precipitation of polypeptides from rotavirus particles grown in the presence of ^{35}S -methionine. The virus particle profile (V) is shown on the left hand side and infected cells (In) are used as marker tracks. The immune precipitates of the various monospecific antisera (α) are labelled at the top. Numbers on the left refer to polypeptides from rotavirus particles and those on the right to virus polypeptides of infected cells.



6.3 Characterisation of the monospecific antisera by indirect immunofluorescence.

Immunofluorescence was used as a method of screening for development of the antibody response, and a method of titration of the potency of the antisera. The use of Evans blue as a counterstain after fluorescent staining gave non-fluorescent regions a dark red appearance while fluorescence was bright green so the contrast was exceptionally good and fluorescence was easily detected. Very weak fluorescence appeared yellow to pale green. Immunofluorescence was more a sensitive screening test than immune precipitation. However, it gave no information on the monospecificity of the antibody. Antibody titres for each of the antisera were determined using $0.5 \log_{10}$ dilutions and are given in table 3. α VP2, α VP6 and α VP7.2 all gave roughly 50% of optimum fluorescence at a dilution of $1/330$. α VP7.1 gave 50% optimum fluorescence at $1/100$, and α VP10 gave 50% optimum fluorescence at $1/33$, although at $1/10$ VP10 showed brighter fluorescence than any other antisera.

The pattern of fluorescence given by each of the monospecific antisera was examined. In each case, the pattern was broadly similar as seen in fig.7. Fluorescence was cytoplasmic, often patchy and most intense in the perinuclear region. Some spots of fluorescence were also seen over the nucleus, especially with α VP6 (fig.7). It is

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TABLE 3
Immunofluorescence with calf rotavirus-infected BSC-1 cells

Serum	Dilution giving optimum fluorescence	Dilution giving $\frac{1}{2}$ optimum fluorescence	Membrane fluorescence [†]	Time of Appearance [*]
α VP2	1/33	1/330	-	8h
α VP6	1/33	1/330	-	8h
α VP7.1	1/10	1/100	-	8h
α VP7.2	1/10	1/330	-	8h
α VP10	1/10	1/33	-	8h

* Cells were examined at 4, 6, 8, 10 and 12h p.i.

† Cells fixed in 3.7% formaldehyde in PBS, and unfixed cells, were used in immunofluorescence to detect membrane antigens.

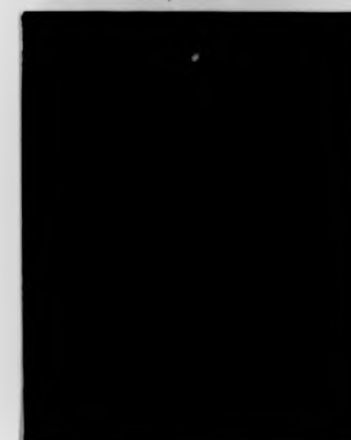
Fig.7 Immunofluorescence of cells infected with calf rotavirus at 5 p.f.u./cell for 19h at 37°C and fixed with methanol at -70°C. Immunofluorescence was carried out by the standard method using a) preimmune serum, b) α VP1, c) α VP2, d) α VP3*,4*, 3) α VP4.2, f) α VP6, g) α VP7.1, h) α VP7.2 j) α VP10 and k) C1 serum.



c) α 2



b) α 1



a) Preimmune



e) α 4.2



d) α 3*,4*

Fig.7 Immunofluorescence of cells infected with calf rotavirus at 5 p.f.u./cell for 19h at 37°C and fixed with methanol at -70°C. Immunofluorescence was carried out by the standard method using a) preimmune serum, b) α VP1, c) α VP2, d) α VP3*,4*, 3) α VP4.2, f) α VP6, g) α VP7.1, h) α VP7.2 j) α VP10 and k) C1 serum.



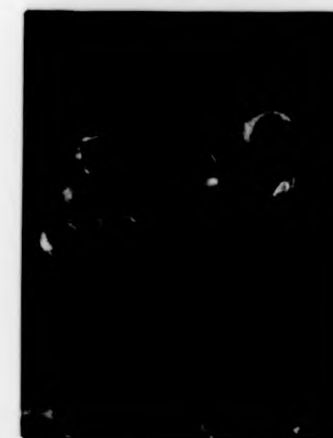
c) α 2



b) α 1



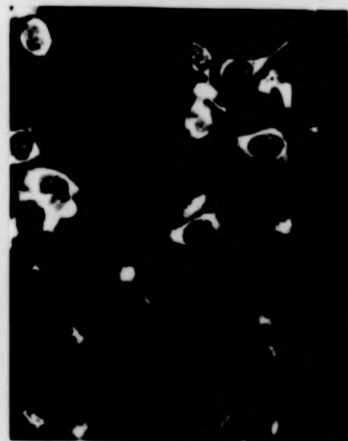
a) Preimmune



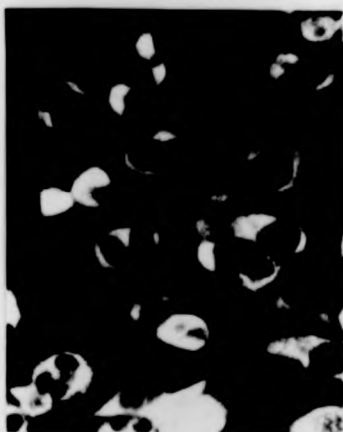
e) α 4.2



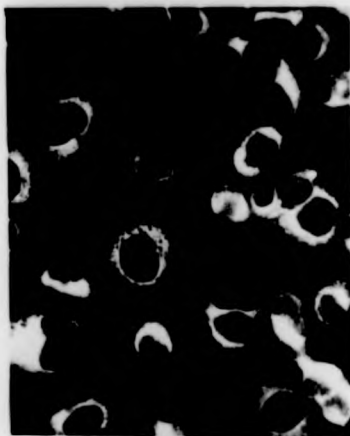
d) α 3*,4*



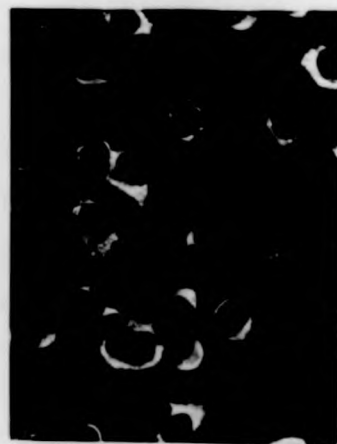
f) $\alpha 6$



g) $\alpha 7.1$



h) $\alpha 7.2$



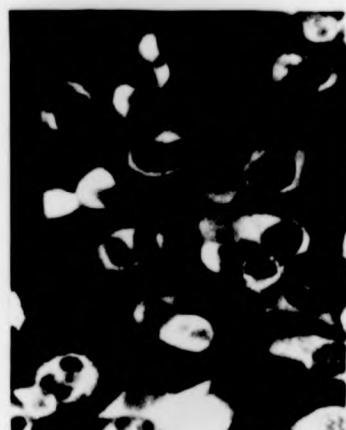
j) $\alpha 10$



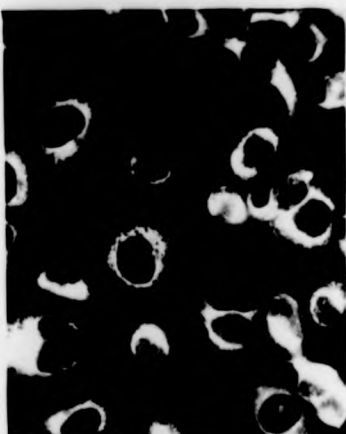
k) C1



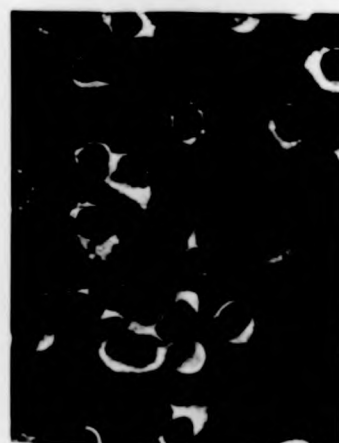
f) $\alpha 6$



g) $\alpha 7.1$



h) $\alpha 7.2$



j) $\alpha 10$



k) C1

6.3 (Cont'd.)

not possible to say if these fluorescent spots are actually in the nucleus or in the cytoplasm covering the nucleus. No membrane fluorescence was detected either in unfixed cells or in cells fixed in 10% formaldehyde (or glutaraldehyde) even though VP7.1, VP7.2 and VP10 are glycoproteins.

The time course of the appearance of virus antigens was also studied. Although rotavirus protein synthesis is detectable by 4h after infection, fluorescence was not easily detectable using any antisera until 8h after infection (table 3). Some weak and pale fluorescence was visible after 6h. It was interesting that all antigens were detectable at the same time, since the synthesis of some antigens detected by incorporation of ^{35}S -methionine into specific proteins is detected earlier than others - notably VP6 which is detectable after 2h (see chapter 4). Cytoplasmic immunofluorescence was optimal at 12h p.i. and this was the time normally chosen for fixation of cells.

6.4 Identification of rotavirus glycoproteins and their precursors by immune precipitation of cells treated with tunicamycin.

Polypeptides VP7, VP7.1, VP10 and VP10c have already been shown to be absent from infected cells treated with tunicamycin (section 4.3, McCrae and Faulkner-Valle, 1981). The purpose of this experiment was to compare precipitation by the antisera VP7.1 and VP7.2 which both precipitate the same polypeptides when there is normal glycosylation. VP7.2 had not been identified as a glycoprotein, so identification of the immune precipitates of α VP7.2 would be expected to shed further light on the relationship of VP7.1 and VP7.2 and to determine whether both were glycoproteins.

Tunicamycin-treated and untreated cells were first precipitated with anti-rotavirus serum G203 [fig. 8(1)]. Precursor proteins VP12 and the broad and indistinct vpr7 (VP12 is a precursor of VP10 and vpr7 is a precursor of VP7) were precipitated from tunicamycin-treated cells but not from untreated cells. The vpr7 precipitated may represent more than one precursor polypeptide for VP7, VP7.1 and VP7.2. In untreated cells VP7, VP7.1 and VP10 were precipitated (more of VP7.1 was precipitated than of VP7 and VP10 was inefficiently precipitated) and more of these three were precipitated from cells treated with tunicamycin. No precipitate of VP7.2 could be seen, as

6.4 (Cont'd.)

this was masked by a strong precipitate of VP8 in cells labelled in the presence and absence of tunicamycin.

Fig.8(11) shows the polypeptides immune precipitated from tunicamycin-treated and untreated cells with pre-immune serum and with α VP7.1 and α VP7.2. Preimmune serum did not precipitate any protein in either case. α VP7.1 and α VP7.2 both precipitated the same two major bands from infected cells without tunicamycin - one migrating in the region of VP7/VP7.1 and the other migrating as expected for VP7.2. Fig. 8(i) suggested that VP7 was almost completely processed to VP7.1 in these cells so the slower migrating band probably represents mostly VP7.1 which is masking VP7. At least one minor polypeptide was also precipitated from these cells (probably vpr7). α VP7.1 and α VP7.2 both precipitated two polypeptides from tunicamycin-treated cells and these resolved as two minor species comigrating with vpr7, but the precipitation was inefficient. These results confirm that VP7.2 is a glycoprotein, and suggest that vpr7 consists of at least two polypeptides which share at least one antigenic determinant with VP7, VP7.1 and VP7.2.

Fig.8

a) Immune precipitation (IP) from rotavirus infected (I), tunicamycin-treated (+Tun) and untreated (-Tun) BSC-1 cells with G203, α VP7.1 or α VP7.2 antisera. Cells infected at 5 p.f.u./cell were incubated at 37°C in the presence or absence of 5 μ g/ml tunicamycin and pulse-labelled with [35 S]-methionine at 6-6.5h post infection. The numbers on the left refer to virus polypeptides in infected cells and numbers and arrows on the right refer to the immune-precipitated polypeptides.

b) Immune precipitation of samples as above with α VP7.1, α VP7.2 or preimmune (P) serum. The numbering system is the same as above.

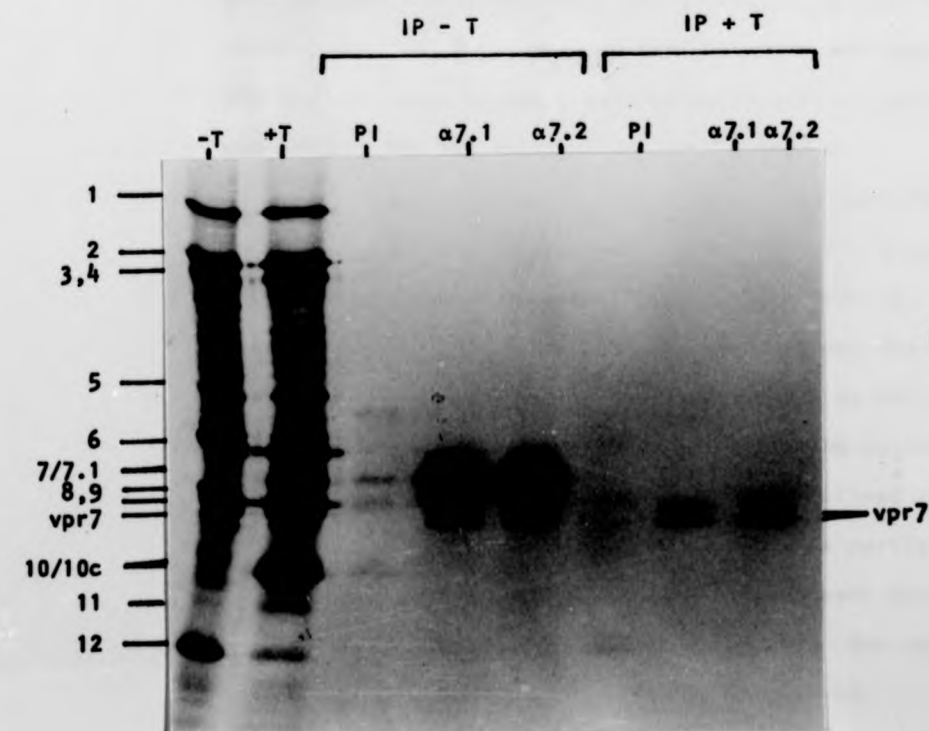
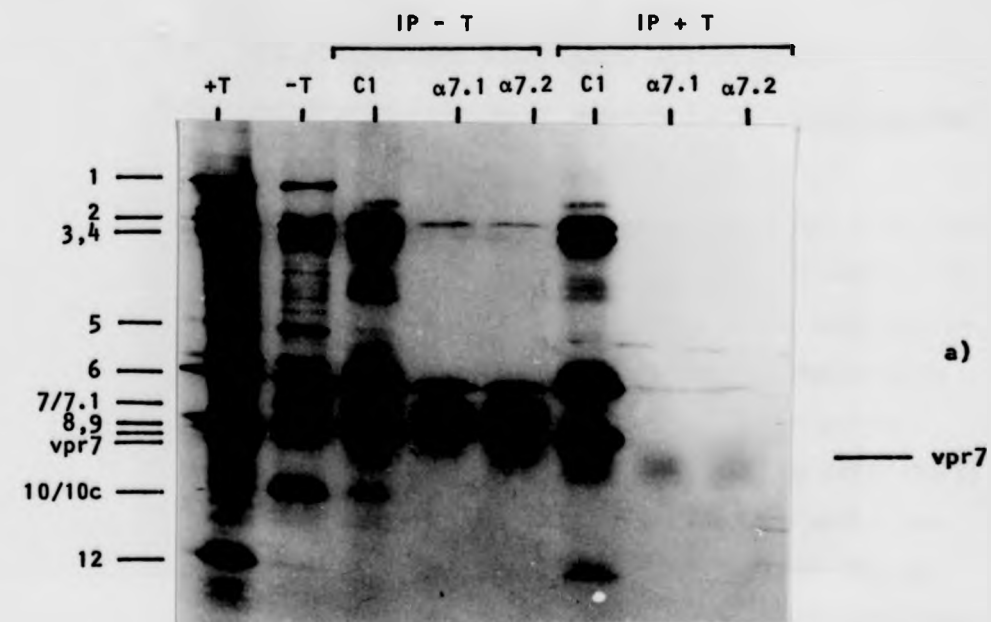
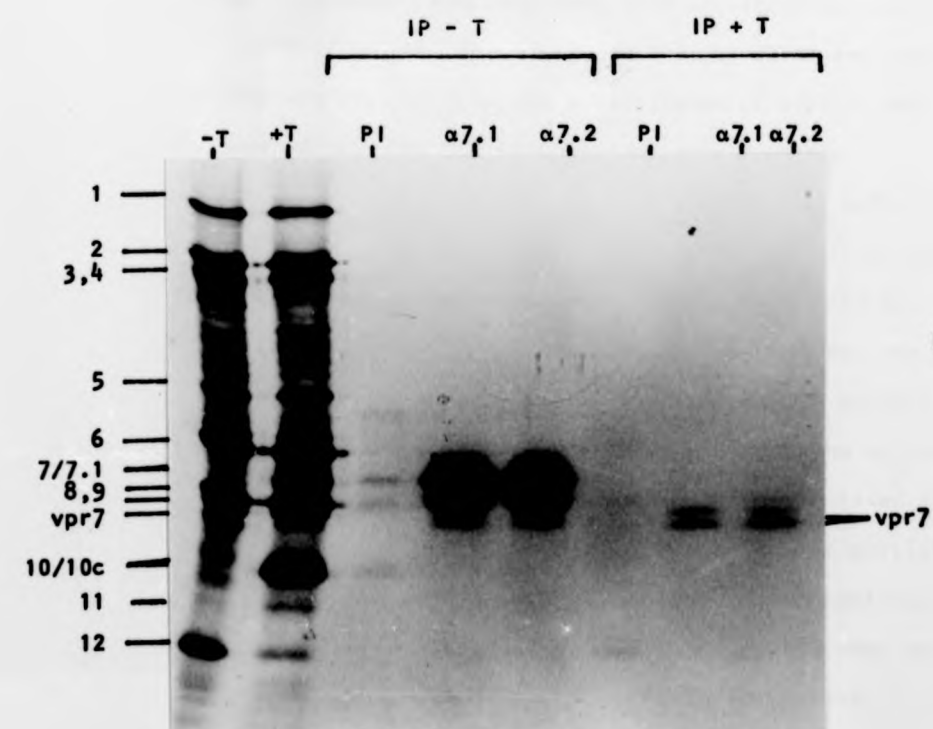
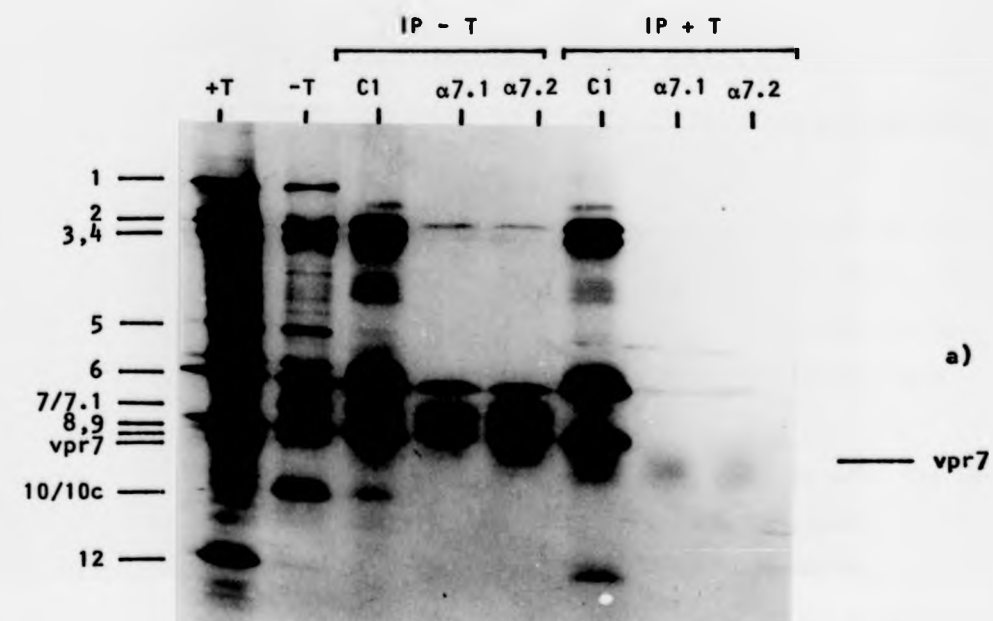


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b) Immune precipitation of samples as above with α VP7.1, α VP7.2 or preimmune (P) serum. The numbering system is the same as above.



6.5 Are the monospecific antisera type-specific?

6.5.1 Immune precipitation of porcine rotavirus polypeptides with monospecific antisera.

Extracts of cells infected with the OSU strain of porcine rotavirus were immune precipitated by α VP2, α VP6, α VP7.1, α VP7.2 and α VP10. These monospecific antisera precipitated the same polypeptides from cells infected with porcine rotavirus as they did with calf rotavirus polypeptides, i.e. VP2 was precipitated by α VP2, VP6 by α VP6 and the VP7 group of polypeptides by α VP7.1 and α VP7.2. VP10 was not precipitated by α VP10 (fig.9). Some of the other polypeptides were also non-specifically precipitated - α VP2 and α VP6 also precipitated some of VP3*4*, VP8, VP2 (by α VP6), and VP6 (by α VP2) and some VP6 was also seen in the precipitates of α VP7.1, α VP7.2 and α VP10. The OSU polypeptides were probably insufficiently disassociated for a completely specific reaction. However, it is evident that none of the monospecific antisera are type-specific as determined by immune precipitation, but this does not rule out the fact that they may have type-specific determinants as well as group-specific determinants. Adsorption of the antisera to the appropriate polypeptide from OSU immobilised to a solid support (rather than adsorption to virus particles in which only part of the polypeptide is exposed) would adsorb out all the group specific antibodies. One could then test for type-specific antibodies by immune

Fig.9 PAGE of immune precipitation of cells infected with OSU porcine rotavirus using monospecific antisera against calf rotavirus.

Infected cells were pulse-labelled from 6 to 6.5h p.i. Tracks show cells infected with calf rotavirus (In) and OSU-infected cells precipitated with the various antisera (α). Virus polypeptide of calf-rotavirus infected cells are labelled on the left and proteins immune precipitated from OSU-infected cells are labelled on the right.

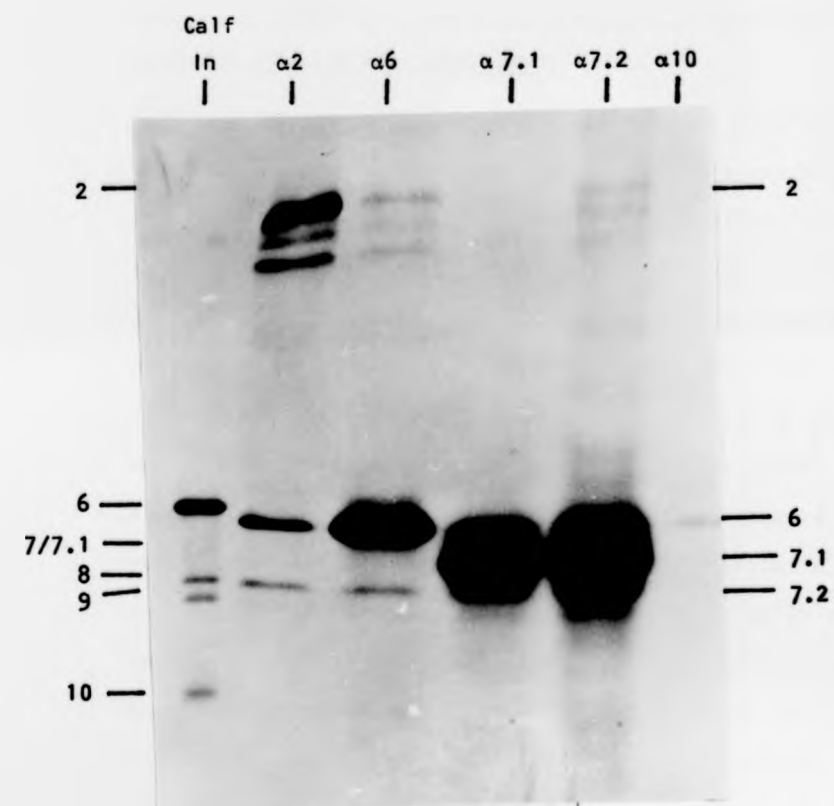
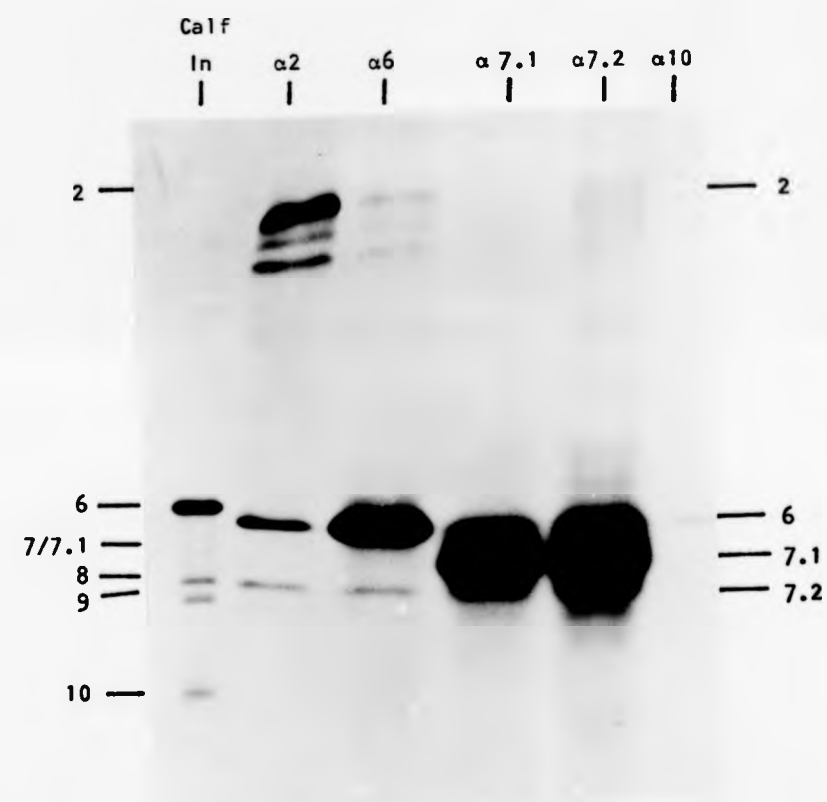


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6.5.1 (Cont'd.)

precipitation of calf rotavirus polypeptides with the unadsorbed serum.

6.5.2 Immunofluorescence of porcine rotavirus infected cells with the monospecific antisera.

Type specificity of the monospecific antisera was also investigated by testing the immunofluorescent reaction of each of them with cells infected with a different rotavirus serotype, i.e. with OSU. α VP2, α VP6, α VP7.1 and α VP7.2 all had a strong immunofluorescent reaction with porcine rotavirus infected cells and only VP10 had a weak immunofluorescent response (fig.10). This could mean that VP10 is a type-specific antigen but could also indicate that OSU synthesises less VP10 than calf rotavirus so that lack of fluorescence may reflect a lack of antigen. However, since OSU-infected cells seen in fig.11 chapter 5 appeared to synthesise at least as much VP10 as cells infected with calf rotavirus and since VP10 is an outer shell glycoprotein apparently involved in neutralization (chapter 7), type specificity of VP10 is strongly suspected. The outer shell glycoproteins VP7.1 and particularly VP7.2 are expected to have type-specific antigenic determinants but may also have a high proportion of group-specific determinants which could mask the type specificity. Denaturation of VP7.1 and VP7.2 prior to immunisation may have

Fig.10 Immunofluorescence of cells infected with porcine rotavirus (OSU) for 19h at 37°C and fixed with methanol at -70°C. a) α VP2, b) α VP6, c) α VP7.1, d) α VP7.2 and e) α VP10, were used in the standard immunofluorescence procedure.

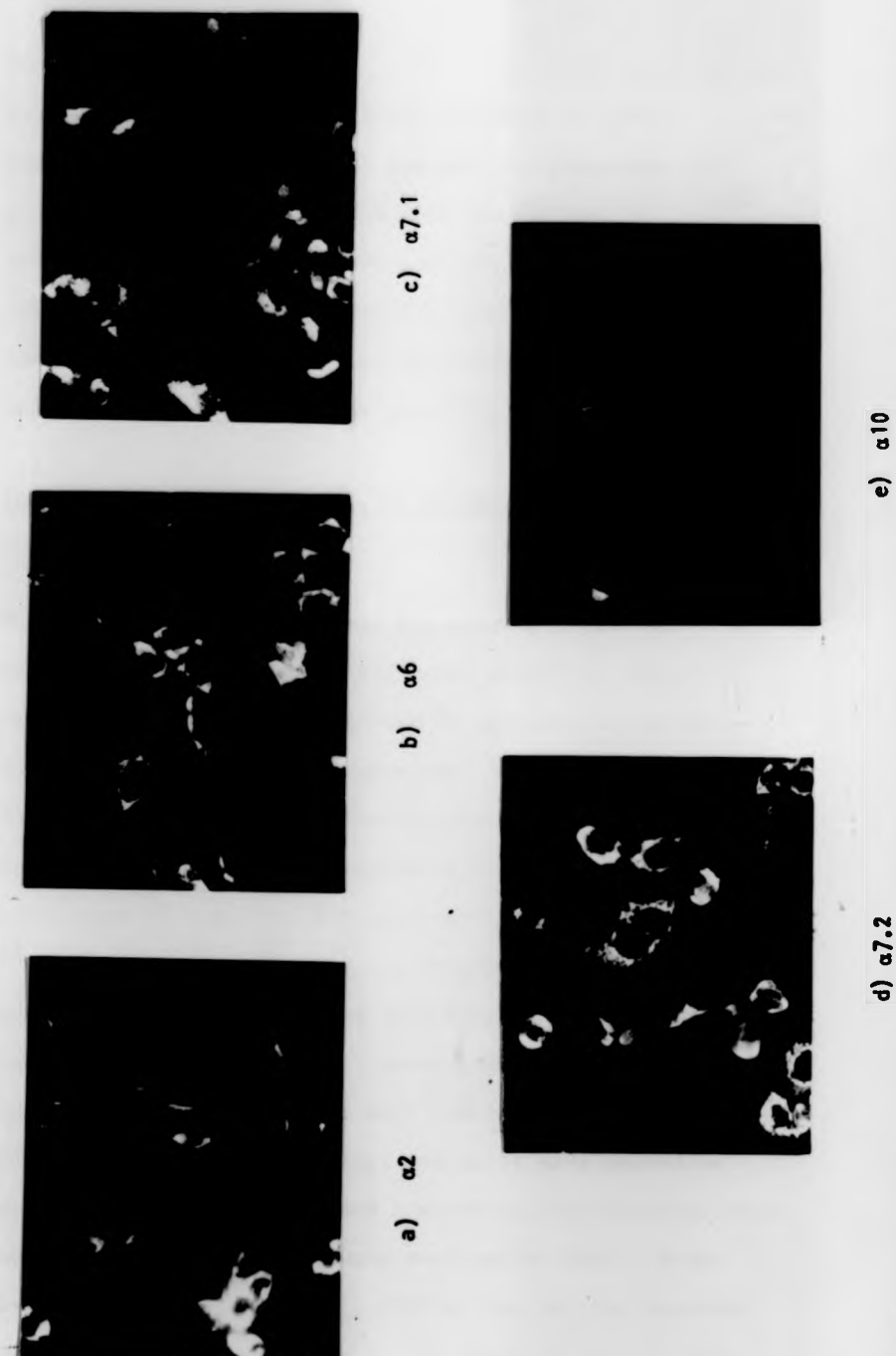
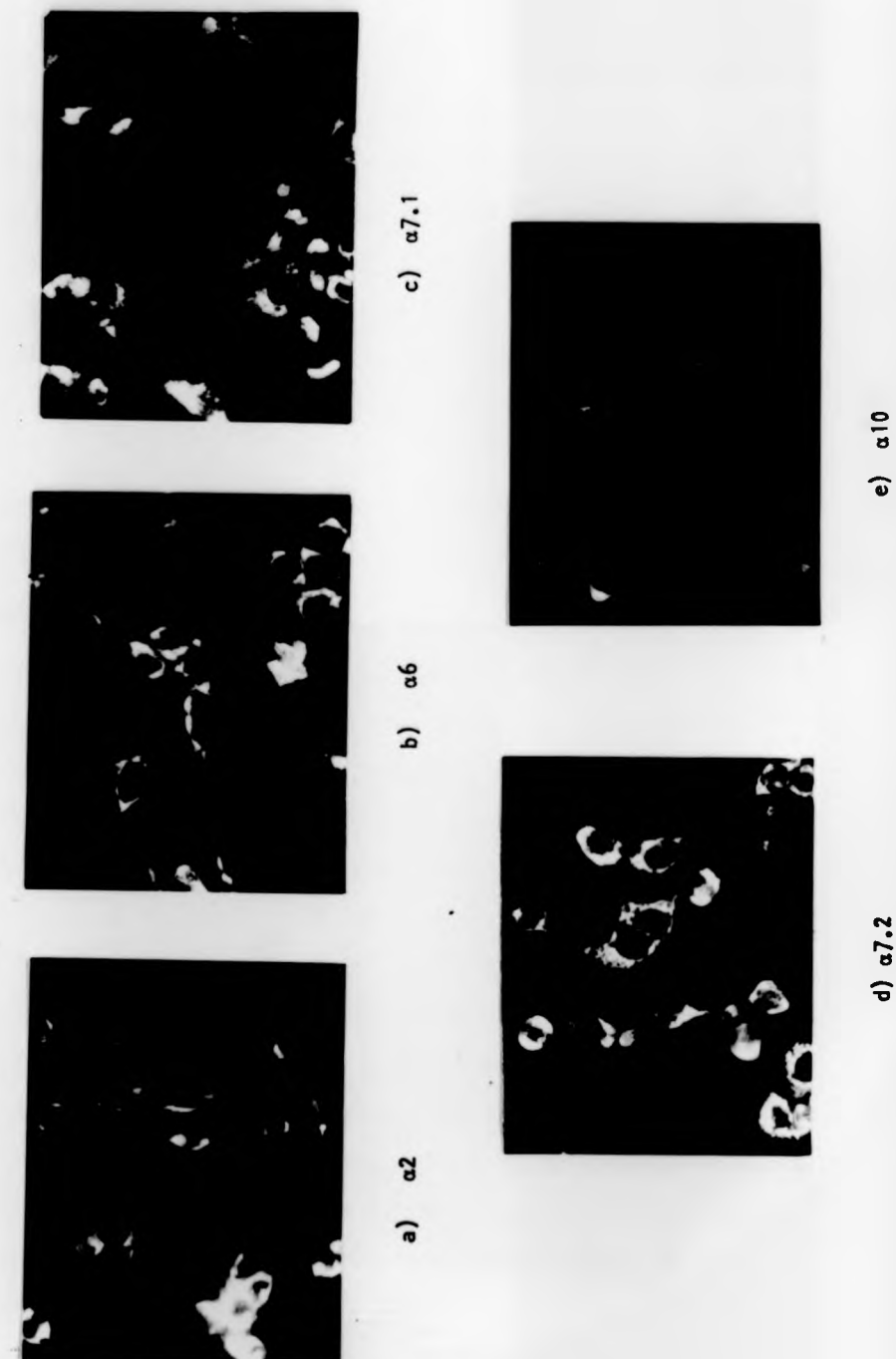


Fig.10 Immunofluorescence of cells infected with porcine rotavirus (OSU) for 19h at 37°C and fixed with methanol at -70°C. a) α VP2, b) α VP6, c) α VP7.1, d) α VP7.2 and e) α VP10, were used in the standard immunofluorescence procedure.



6.5.2 (Cont'd.)

destroyed their type-specificity and most of α VP7.1 and α VP7.2 may be antibodies against the group-specific determinants, although α VP7.2 does neutralize infectivity (chapter 7). α VP2 and VP6 are inner shell polypeptides but VP6 has subgroup specificity (Kalica et al., 1981). These two polypeptides may also have group-specific and type-specific determinants.

6.6 Identification of the rotavirus protein that binds to cells.

The virus protein responsible for binding rotavirus particles to cells was identified by adsorbing radio-labelled proteins which are free in cytoplasmic extracts (hence no denaturation is necessary) to cell monolayers. The experiment was carried out as described for reovirus by Lee et al. (1981b). Cytoplasmic extracts were prepared from rotavirus infected cells labelled with ^{35}S -methionine for 6-6.5h p.i., diluted with an equal volume of GEM + 10% FCS and centrifuged at 45,000 r.p.m. for 1h to remove aggregates. After 200 μl of the supernatant was adsorbed to cell monolayers for 1.5h at 4°C with intermittent rocking, the cells were washed 5x in Earle's saline, lysed, and the radioactive proteins which had attached to the cells were analysed by PAGE. Under these conditions, Lee et al. (1981b) did not see any host proteins bound to the monolayers. As shown in fig.11, only

6.6 (Cont'd.)

one rotavirus protein bound to the monolayers. This protein comigrated with VP7.2 or VP8. Purified virus was shown to compete with the binding of this protein to cells - when the cell lysates were mixed with 200 µg/ml purified virus before adsorption, no labelled protein was seen bound to the cells (fig.11). These preliminary results show that either VP7.2 or VP8 bound to the cellular receptor for rotavirus. As VP8 is non-structural, VP7.2 is probably the protein involved. A longer exposure of the gels would show if other rotavirus proteins also bound to the cells, but as VP7.2 was a minor protein very few c.p.m. bound to the cells - hence the low exposure. This binding experiment was done once only, but the lysed cells were run on two gels and the same result was seen in both.

Fig.11 Identification of the rotavirus protein which binds to cells.

Cytoplasmic extracts were prepared from infected cells which had been labelled with ^{35}S -methionine 6 to 6.5h p.i. The extracts were adsorbed onto cell monolayers for 1.5h at 4°C and the cells were then washed extensively, subjected to PAGE and analysed by autoradiography to identify radiolabelled proteins bound to the cells. The extracts were also mixed with unlabelled purified virus particles prior to adsorption to see if virus competes with the free protein for the cell receptor. Tracks on the left and right show infected cell marker tracks. Cells incubated with the extract alone are labelled Cells + E, and cells incubated with the extract and with virus are called Cells +E+V. Virus proteins are labelled on the left and right of the photograph.

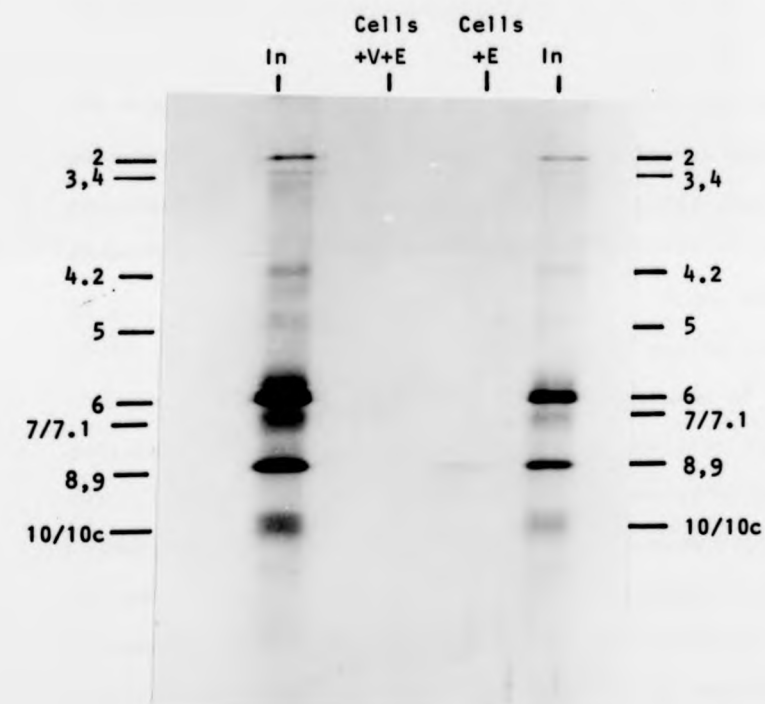
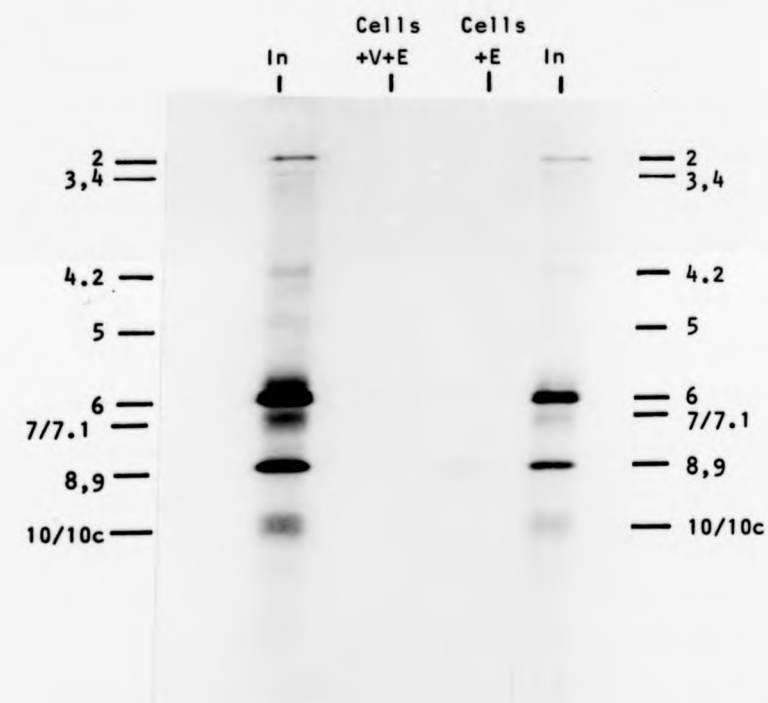


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CHAPTER 6

DISCUSSION

Monospecific antisera were raised against several rotavirus polypeptides and were used to provide substantial information about rotavirus polypeptides in infected cells, including the identification of precursors of certain proteins and confirming the relationship between polypeptides.

The induction of an antibody response to denatured and reduced polypeptides excised from polyacrylamide gels has been described in many virus systems including herpes simplex virus (Powell et al., 1974), foot and mouth disease virus (Bachrach et al., 1975; Melen et al., 1979) and SV40 (Lane and Robbins, 1978), and the antibody response was sufficient to indicate that at least some important immunogenic features of the polypeptides had been retained (e.g. type specific regions and sites involved in neutralization). The quantity of immunogen needed to raise antibody was reported to be much higher than the quantity of native antigen. This was probably due to loss of immunogenicity of the polypeptide during preparation for electrophoresis. We found the task of raising monospecific antisera to rotavirus polypeptides was much more difficult than anticipated, and a prolonged injection regime was needed (table 1). Indeed, after 7 or 8 injections the antibody response to VP1, VP3*,4* and VP4.2 was still very poor.

The two screening systems used for the antisera complemented each other satisfactorily. Immunofluorescence was a fast,

simple and sensitive test, and preliminary tests showed that anti-guinea pig FITC - conjugated antisera did not contain anti-rotavirus antibodies and neither this nor control antisera induced fluorescence. Immunofluorescence was also useful for titration of the antisera. However, one drawback of immunofluorescence is that it does not distinguish between our antibody response to the injected polypeptides or one which may have been acquired through rotavirus infection. Immune precipitation, although slower and marginally less sensitive, was invaluable for determining the specificity of the antisera and the quantity of protein precipitated was easier to judge by eye than the intensity of fluorescence.

Good antibody responses to VP2, VP6, VP7.1, VP7.2 and VP10 were obtained while those to VP3*,4*, VP4.2 and VP1 were weak. VP3*,4* and VP1 are minor virus polypeptides but VP4.2 is relatively well represented in virus particles so we did expect an antibody response to it. It is also thought to be the important trypsin cleavage product of VP3 (Estes et al., 1981), which has been identified as the viral haemagglutinin, and important for growth and plaque formation in tissue culture (Greenberg et al., 1981; Kalica et al., 1983), so it must have an important role in the infection of cells. An antibody response to VP10 was interesting as some groups do not recognise its existence in virus particles (Estes et al., 1981; Novo and Esparza, 1981).

The monospecificity of the antisera was primarily determined by immune precipitation using cell extracts pretreated with 2%

SDS and 5% BME at 100°C for 2 min as this treatment helped to disaggregate complexes and prevent non-specific co-precipitation of polypeptides. In the absence of the pretreatment step, VP2 and VP6 were non-specifically precipitated to the greatest extent as these two polypeptides are abundant in infected cells and are major constituents of immature virus particles that have been observed in infected cells (Saif *et al.*, 1978) and therefore likely to be precipitated by antibodies to other antigens in these particles. Monospecificity (i.e. specific for only 1 protein) of α VP2 and α VP6 was confirmed, as they reacted with only VP2 and VP6 respectively, and was also confirmed for α VP3*,4*. α VP3*,4* precipitated only VP2 probably because VP3*,4* are only present in minor quantities in infected cells or virus (fig.1). VP7.1 and VP7.2 were either immunologically related or always aggregated together as their antisera both precipitated these two polypeptides (and their precursors which were identified by the use of tunicamycin). Single dimension peptide mapping of these two polypeptides by Espejo *et al.* (1981) has confirmed their relationship. Immune precipitation of tunicamycin-treated cells by α VP7.1 and α VP7.2 showed that vpr7 and not VP12 was precursor of VP7, VP7.1 and VP7.2, a fact which had not previously been determined. The lack of precipitation of VP10 by α VP10 was surprising because VP10 was a very reactive antiserum by IF, indicating that both antigen and antibody were present. Presumably even the mildest treatment with detergent (0.5% NP40) destroyed the relevant antigenic determinants or prevented their availability to antibody by

coating (see chapter 5). However, since denatured VP10 excised from gels is immunogenic, we conclude that the lost antigenic determinants are reconstituted after PAGE or perhaps after injection into the guinea pigs. This could be by refolding of protein or loss of detergent from the surface. Another possibility is that VP10 in infected cells is closely associated with the membranous inclusions seen in infected cells (Chasey, 1977) and that these are not solubilised by 0.5% NP₄₀. Possibly higher concentrations of NP₄₀ are needed for preparation of the cell extract. Alternatively, as suggested in chapter 5, less NP₄₀ or replacement of it by SDS and DOC may prevent the hydrophobic regions of the protein from becoming coated with micelles of the non-ionic detergent and thus masked from antibody. Unfortunately the lack of precipitation of VP10 by α VP10 means that the specificity of this antiserum could not be determined.

Immunofluorescence was carried out using each antiserum in order to give an indication of the cellular location of each of the antigens at various times after infection. For example, in most enveloped virus systems the glycopolypeptides are known to be processed in the cell membranes and are detectable on the cell surface, and some virus polypeptides such as the NP and NS1 of influenza virus and many structural proteins of herpes simplex virus among others are associated with the nucleus (Hudson et al., 1978; Spear and Roizman, 1968). In

the case of rotavirus, however, the fluorescence pattern was very similar with each antiserum - it was cytoplasmic and characteristically perinuclear and patchy. This reflects the observed pattern of rotavirus replication in infected cells where viruses are concentrated in areas of viroplasm near the RER and mature by budding into the RER (Chasey, 1977, 1980; Altenberg et al., 1980). Petrie et al. (1982) have used monospecific antisera against VP2, VP6 and VP7.1 in ultrastructural studies by immunoperoxidase staining and electron microscopy and found that α VP2 and α VP6 labelled cytoplasm as well as areas of viroplasm and RER containing virus particles, while α VP7.1 labelled only virus particles enclosed in RER but not in viroplasm. They concluded that VP2 and VP6 are synthesised throughout the cytoplasm and become concentrated in viroplasmic inclusions while VP7.1 is synthesised in the RER and viruses appear to acquire VP7 while budding into the cisternae of RER. We also observed fluorescent spots in the region of the nucleus, most markedly with α VP6. These may correspond with tubular forms of rotavirus which have been observed in the nuclei of tissue culture cells and of cells in sections of villi infected with porcine and murine rotavirus (Pearson and McNulty, 1979; Banfield et al., 1978; Salf et al., 1978; Altenburg et al., 1980). The diameter of these tubules was not wider than that of single shelled particles and, as VP6 is the major component of single shelled particles, α VP6 may detect these viral structures in nuclei. No rotavirus

antigen could be detected in the plasma membrane by any of the antisera in live cells (i.e. unfixed) or in cells fixed with formalin to preserve the cellular membranes (Sternberger, 1979). It therefore appears that rotavirus glycoproteins are not processed on the plasma membrane. This is plausible as rotaviruses do not acquire a cell-derived outer membrane and as rotaviruses are associated with discrete areas of viroplasm and the RER. Glycoproteins may associate with the membranes of the RER and transfer to rotaviruses as they bud into cisternae of RER. No changes in the pattern of fluorescence were detected by the monospecific antisera over a time course. It appears that the pattern of replication is relatively simple in that all polypeptides remain in the same area of the cells throughout infection. The monospecific antisera VP2, VP6, VP7.1 and VP7.2 cross-reacted with cells infected with porcine rotavirus OSU, but the fluorescence seen with α VP10 was weaker in cells infected with porcine rotavirus than in calf rotavirus infected cells. We expected α VP7.1 and α VP7.2 to be type-specific; however, the cross-reactivity of these antisera could be due to the fact that they were raised against denatured and reduced polypeptides in which group specific determinants normally hidden were exposed or type-specific determinants had changed conformation. VP10 is either more completely type-specific or more likely to renature after denaturation. VP6 was also expected to have specific antigenic determinants as it has been used for determining subgroup specificity (Kalica *et al.*, 1981). These antigens could have type-specific determinants which are not detected due to the fact that they also have group-specific determinants.

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Finally, the result that a protein comigrating with VP7.2 or VP8 bound to cells suggests that VP7.2 is involved in attachment of virus to cellular receptors since VP8 is a non-structural protein. The binding is highly specific since VP7.2, which is difficult to detect in cells and is a minor component compared to VP7 or VP7.1, was the only protein identified. This result is compatible with those of Matsuno and Inouye (1983), who found their antiserum against VP7.1/VP7.2 inhibited binding of the virus to cells. However, they did not compare this result with any other monospecific antisera, and the exposure of our gel was low so it does not rule out the possibility that other proteins may also be involved. The inhibition seen by Matsuno and Inouye (1983) was relatively low (68-80%). A similar experiment tried with our antisera which had lower antibody titres than those of Matsuno and Inouye (1983) gave very low levels of inhibition (data not shown). The fact that VP7.2 is seen binding to cells and not VP7.1 suggests that VP7.2 contains antigenic determinants not expressed on VP7.1 (see also chapter 7) and suggests that these proteins are both seen on each virus particle (see also chapter 4).

CHAPTER 7

CHAPTER 7

Identification of monospecific antisera that react with rotavirus particles.

INTRODUCTION

This chapter extends the work described in chapter 6. The monospecific antisera are characterised by their reactions with rotavirus particles, i.e. their ability to bind to and agglutinate rotavirus and to neutralize rotavirus infectivity are investigated.

7.1 Optimisation of conditions of neutralization as measured by a plaque reduction assay.

We used the rotavirus plaque assay, in which the infectivity of the virus inoculum is activated by trypsin but where the overlay contains no trypsin, as described by Faulkner-Valle et al. (1982). Thus, the low dilutions of the virus inoculum contain more residual trypsin from the virus stock than the higher dilutions. In a preliminary neutralization experiment, the virus titre was reduced more than 50% by low dilutions of all the monospecific antisera tested. It seemed unlikely that all monospecific antisera would neutralize rotavirus infectivity and another explanation was that this plaque reduction was due to the

7.1 (Cont'd.)

serum proteins blocking the enhancement of virus infectivity by trypsin. This alternative was investigated by removing the virus inoculum after adsorption and washing the monolayer several times before overlaying with agar. This treatment should remove trypsin in the inoculum and if the hypothesis is correct a stable, lower titre should be reached. Table 1 shows that this is indeed the case, and the virus titre dropped 50% when cell monolayers were washed several times before being overlaid with agar. For subsequent experiments, cells were washed three times after adsorption to remove any trypsin and serum, and prevent non-specific loss in virus titre with antiserum.

7.2 Plaque reduction assay with each of the monospecific antisera

Antisera that neutralize calf rotavirus infectivity were identified by plaque reduction assay. Table 2 summarises the results of two independent assays using all the monospecific antisera. α VP7.2 neutralized the viral infectivity most efficiently of all the monospecific antisera. Convalescent anti-rotavirus serum (G203) neutralized 99.9% of the viral infectivity, while α VP7.2 always neutralized 98.9-99.7% (tables 2 and 3) and α VP6 always neutralized 50% (table 2). The results with α VP10 were more variable as seen in table 3, even though it always reduced rotavirus infectivity. Usually plaque reduction was 80-93% but it has been as low as 62%. Table 3 shows that neutralization by α VP7.2 only varied

TABLE 1

Effect of removal of residual trypsin on virus titre⁺

Virus Sample	No. of washes	Titre (p.f.u./plate)*
Virus only	0	48
	1	38
	2	32
	3	20
	4	18
Virus + α VP7.1 (1/50)	0	31
	1	20
	2	18
	3	21
	4	23.3

+ Virus inoculum of 50 p.f.u./plate contained a total of 0.01 pg/ml trypsin (diluted from the original virus stock containing 10 μ g/ml). Virus or virus and antiserum (α VP7.1) were adsorbed onto cells for 1h at 37°C. After removal of residual virus, cells were washed 0, 1, 2, 3 and 4 times to remove trypsin and serum.

* Titres = average of two plates.

TABLE 2

Neutralization of rotavirus infectivity with all
the monospecific antisera

SERUM	Titre [#] (p.f.u./plate)		% neutralization	
	Expt. 1	Expt.2	Expt.1	Expt.2
NONE	50	4×10^4	0	0
PREIMMUNE (GP3)	55	3×10^4	0	0
α VP1	53	ND ⁺	0	-
α VP2	42	3×10^4	0	0
α VP3*, 4*	64	ND	0	-
α VP4.2	46	ND	0	-
α VP6	20	2×10^4	60	50
α VP7.1	46	3×10^4	0	0
α VP7.2	0.5	1×10^2	99	99.7
α VP10	6	2×10^3	88	95
G203 ϕ	-	3×10^1	-	99.9

Neutralization was carried out with a low dose of virus (50 p.f.u./plate, expt.1) and a high dose of virus (4×10^4 p.f.u./plate, expt.2) and serum diluted 1/20. Virus and antiserum were mixed for 2h at 25°C and infectious virus titrated as in a plaque assay.

Titres = average of two plates

+ ND = not done

ϕ G203 is a convalescent calf antiserum (1/20) to the strain of rotavirus used in this study.

7.2 (Cont'd.)

0.8% while that found with α VP10 varied 33%. Because α VP6 and α VP10 neutralize rotavirus infectivity less efficiently than α VP7.2, we suggest that VP7.2 is the major antigen responsible for eliciting neutralizing antibody. None of the other antisera neutralized the infectivity of calf rotavirus. It is particularly interesting that α VP7.1, which precipitates the same pattern of polypeptides as α VP7.2 in immune precipitation, did not neutralize, in any of several separate experiments. The titre of α VP7.1 by immunofluorescence was only $\frac{1}{2}$ log dilution less than the titre of α VP7.2, and more α VP7.1 than α VP7.2 bound to virus (see section 7.8). These results indicate that α VP7.2 must contain antibody specific for a determinant present on the major neutralization specific antigen of calf rotavirus, and that this antibody is absent from α VP7.1.

7.3 Titration of antisera by neutralization

Determination of the dilution of antiserum which reduces viral infectivity by 50% is a standard method of titrating antisera. At the lowest dilution used ($1/20$) α VP6 only reduced the titre of calf rotavirus by 50% (so its titre is ≤ 20). Preimmune serum (GP3), α VP7.2 and α VP10 were titrated to determine their neutralization titres. GP3 did not neutralize infectivity at a dilution of $1/20$, α VP7.2 neutralized 50% of the infectivity at a dilution of $1/640$ and α VP10 at $1/160$

TABLE 3

Variation of the degree of neutralization
caused by α VP7.2 and α VP10.

	% neutralization by:-	
EXPT.NO.	α VP10	α VP7.2
1	88	99
2	70	99.7
3	62	99.2
4	95	99.4
5	88.6	98.9
% variation =		33% 0.8%

The percentage neutralization of virus infectivity
by α VP10 and α VP7.2 in each of a number of
experiments are outlined in this table to demonstrate
the high variability of neutralization caused by
 α VP10.

7.3 (Cont'd.)

(table 4). These results also confirm that α VP7.2 has greater neutralizing activity than any of the other antisera prepared in guinea pigs, while its titre measured by immunofluorescence is the same as that of α VP6. The IF titre of α VP7.2 is 1 log higher than that of α VP10. Thus, it could be said that α VP10 is as important in neutralization as α VP7.2.

7.4 Time course of neutralization with α VP7.2.

This experiment was done to determine the time-dependent properties of neutralization with α VP7.2. The experiment was not done with α VP10 or α VP6, because of the low and variable nature of their neutralizing properties. Fig.1 shows that neutralization of infectivity with α VP7.2 at this dilution is time dependent. On a semi-log plot shown in fig.2 the slope is linear for more than 105 min. G203 under the same conditions takes 15 min for the same loss of infectivity and is therefore a more potent antiserum than α VP7.2. The neutralization curve seen in fig.1 follows the general pattern described for animal viruses (Della-Porter and Westaway, 1977). A lag phase of 5 min. duration occurs followed by a first order kinetic phase for 105 min. and a persistent fraction of 10^2 p.f.u. remains (from 3×10^5 p.f.u.). This experiment therefore shows that the monospecific antibody behaves as a neutralizing antibody by accepted criteria (Della-Porter and Westaway, 1977).

TABLE 4

Titration of the neutralizing activity of the
monospecific antisera by plaque reduction.

Serum	1/Dilution	Titre p.f.u./plate ⁺	Titre of Serum
-	-	51	<20
PREIMMUNE	20	55	
(GP3)	80	47.5	
α VP10	40	6	160
	160	24.5	
	640	56.5	
	2560	55.5	
α VP7.2	20	0.5	640
	40	1	
	160	0.5	
	640	22	
	2560	47.5	

Virus (50 p.f.u./plate) was mixed with antisera diluted as indicated above for 2h at 25°C, and then titrated by plaque assay.

⁺Titre = average of two titrations

Fig.1

Time course of virus neutralization.
Virus (3×10^5 p.f.u./ml) was mixed with
preimmune serum (○) or α -VP7.2 (●) both
diluted 20-fold and incubated at 25°C.
Samples (0.1 ml) were withdrawn at
intervals, diluted $1/100$ and immediately
titrated by plaque assay.

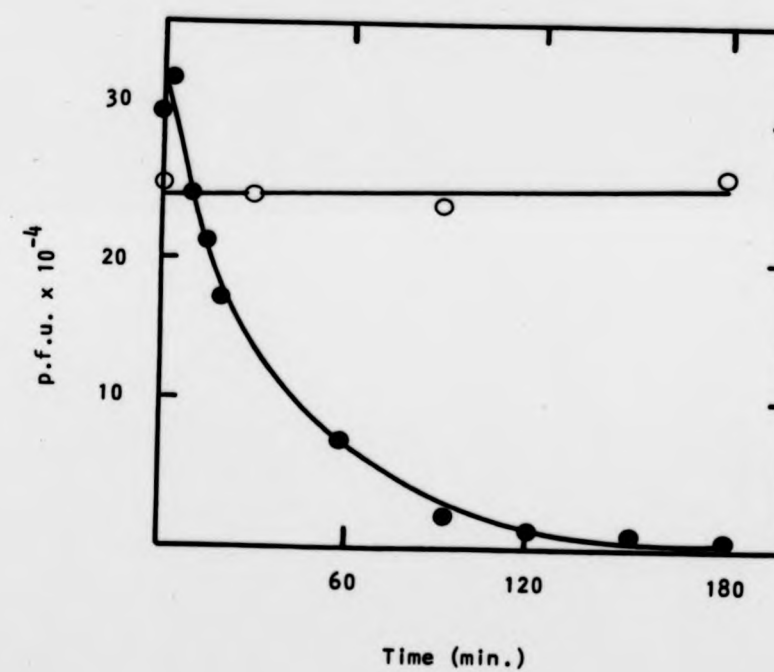
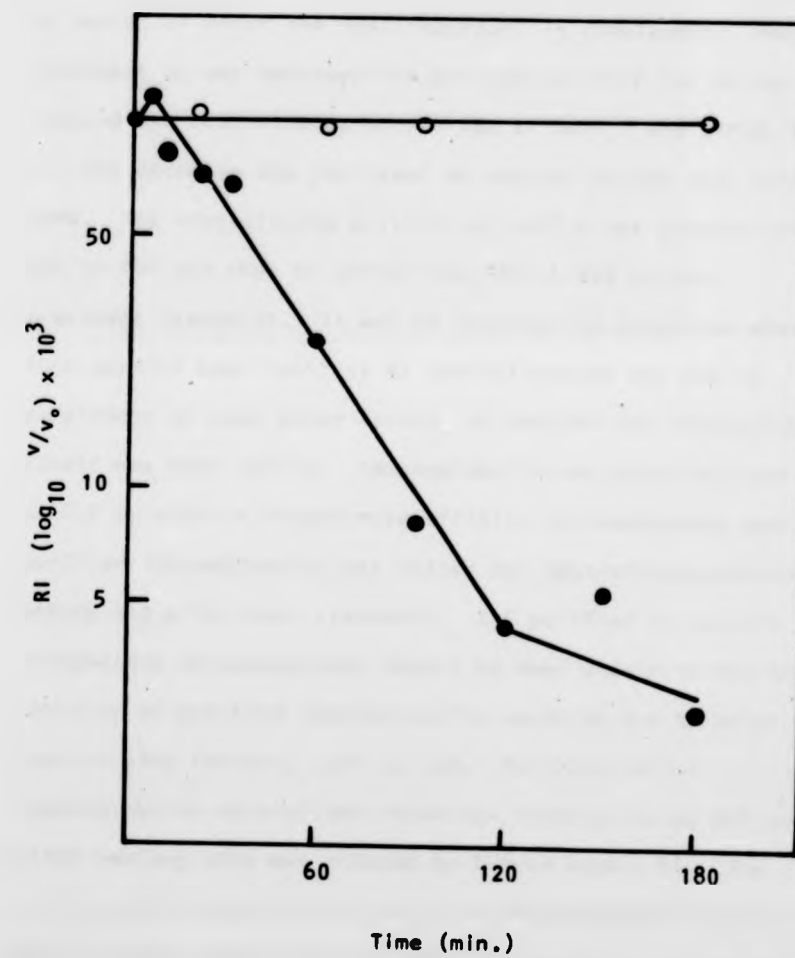


Fig.2 Time course of virus neutralization.
The same time course shown in fig.1
is plotted as \log_{10} p.f.u. against
time.



7.5 The effect of heat treatment on the neutralization activity of our monospecific antisera.

Heat treatment of antisera at 56°C for 30 min is a common method of destroying non-specific neutralizing factors in serum, of which the most important is complement. Heat treatment of our monospecific antisera at 56°C for 30 min reduced the neutralizing activities of α VP7.2 and α VP10, but did not decrease the low level of neutralization seen with α VP6. The neutralizing activity of α VP7.2 was reduced from 99% to 80% and that of α VP10 from 90% to 60% by heat treatment (table 5). It was of interest to determine whether this partial heat lability of neutralization was due to complement or some other factor, or whether the immunoglobulin itself was heat labile. Immunoglobulin was purified from α VP7.2 by protein A-sepharose affinity chromatography and the purified immunoglobulin was tested for neutralizing activity before and after heat treatment. IgG purified by protein A-sepharose chromatography should be heat stable so any heat lability of purified immunoglobulin would be due to other neutralizing factors, such as IgM. Purified α VP7.2 immunoglobulin neutralized rotavirus infectivity by 93% and after heating this was reduced by 2-fold (table 6). The relative heat stability of purified immunoglobulin suggests that the heat lability of the neutralizing activity of the α VP7.2 serum is due to some component other than immunoglobulin. The effect of complement on neutralization of infectivity by heat treated antiserum was next investigated.

Table 5. Neutralization by heat treated antisera

Serum	Titre ⁺ p.f.u./plate	% Neutral- ization
-	148	-
α VP7.2	2	98.6
α VP7.2 (heat treated)*	35	76.4
α VP10	15	89.9
α VP10 (heat treated)	58	61.0

Virus and antisera were incubated for 2h at 25°C, and titrated by plaque assay.

* Titre = average of two titrations.

* Antisera were heated at 56°C for 30 min.

Table 6 Neutralization with purified Immunoglobulin

Immunoglobulin	Titre p.f.u./plate	% Neutral- isation.
-	148	-
α VP7.2	10.5	92.9
α VP7.2 (heat treated)	26.5	82.1
α VP10	61.5	58.4
G203	0	99.3

Neutralization was as described previously, except that purified Immunoglobulin (diluted $1/10$) was used instead of antisera.

7.5 (Cont'd.)

Commercial complement in the form of lyophilised guinea pig serum was added to heat treated antiserum, and was also tested for neutralizing activity on its own (table 7). The addition of complement to heat treated antiserum completely restored its neutralizing activity, indicating that neutralization of calf rotavirus by α VP7.2 is enhanced by complement but is not completely dependent on it. The effect of complement on neutralization by α VP10 was not tested, as the natural variation in neutralization by this antiserum was as high as the difference in titre between heat treated and non-heated α VP10.

7.6 Effect of ultrasonication of neutralized virus before titration.

This experiment was designed to determine whether any or all of the neutralizing activity of either of the monospecific antisera, α VP7.2 or α VP10, was 'pseudo-neutralization' due to aggregation of virus particles by antibody. Neutralized virus was subjected to ultrasonication before titration, both with gentle sonication by immersion in a sonibath for 1 min and by harsher sonication with a soniprobe for 10 sec. Both ultrasonication treatments reduced the titre of non-neutralized virus (table 8) by 1.8 fold and 15 fold respectively. However, sonication did not increase the infectivity of virus neutralized by

Table 7 Effect of complement on neutralization with heat treated antisera.

Serum	p.f.u./ plate	% Neutral- ization
-	160	-
α VP7.2 (heat treated)*	15.5	90.3
α VP7.2 (heat treated + C') ⁺	1	99.3
α VP7.2 (untreated)	1.5	99.1
C' alone	152	<10

* Neutralization and heat treatment were performed as described previously.

⁺ Lyophilised complement was restored as directed and diluted $1/14$.

TABLE 8 Effect of ultrasonication on the infectivity of
neutralized virus*

Serum	No Sonication		Sonibath		Soniprobe	
	Titre [†]	% neut.	Titre	% neut.	Titre	% neut.
None	1.2×10^3	0	6.7×10^2	0	8×10^1	0
α VP7.2	1.3×10^1	98.9	6.5×10^0	99	1.5×10^0	98.1
α VP10	1.3×10^2	88.6	6.9×10^1	89.8	1.4×10^1	82.5

* Virus was incubated without serum or with α VP7.2 or α VP10 diluted $1/20$ for 2h at 25°C. Samples were subjected to no treatment, 1 min in a sonibath, or 10 sec with a soniprobe and the residual virus was titrated.

† Titre = p.f.u./sample.

Samples were titrated after 10x and 100 x dilution.
(the average of 2 plates was taken).

7.6 (Cont'd.)

either α VP7.2 or α VP10, so neutralization of these viruses by α VP7.2 and α VP10 does not occur by formation of aggregates.

7.7. Do mixtures of monospecific antisera have synergistic effects with one another on neutralization of rotavirus?

Synergistic effects of antibodies have been reported for the neutralization of many viruses including adenovirus, Rous sarcoma virus and a population of mixed particles of vesicular stomatitis virus and parainfluenza virus SV5 (Daniels, 1975; Della-Porter and Westaway, 1978). In the case of adenovirus, neutralization is greater when a mixture of antibody against hexons and pentons or of antibody against hexons and fibres was used than when either antibody in each combination is used alone.

We tested for the possibility of synergistic neutralization of rotavirus by combinations of rotavirus monospecific antisera. Antisera which had some neutralizing activity were mixed in pairwise combinations keeping the concentration of serum the same as in assays with only one type of antiserum. A mixture of the neutralizing α VP7.2 and the non-neutralizing α VP7.1 was also tested for synergistic effects. None of the combinations of antiserum neutralized rotavirus more efficiently than α VP7.2 (table 9). In fact, neutralization was 10-fold lower with a mixture of α VP7.2 and α VP6 than with α VP7.2 alone,

TABLE 9

PLAQUE REDUCTION WITH MIXED ANTISERA

Serum	Titre (p.f.u./plate)	% Neutral- ization
NONE	3.6×10^4	0
α VP7.2	1.3×10^2	99.6
α VP7.1	3.2×10^4	11.2
α VP6	1.6×10^4	55.6
α VP10	2.4×10^3	93.3
α VP7.2 + α VP7.1	9.5×10^1	99.7
α VP7.2 + α VP6	9.8×10^2	97.3
α VP7.2 + α VP10	1.8×10^4	50
α VP10 + α VP6	4.0×10^3	88.9

Neutralization with one antiserum or two mixed antisera was as described previously. Antisera were diluted 1/20, but in the case of mixed antisera each was diluted 1/40. (Note that α VP7.2 neutralizes as efficiently at a dilution of 1/160 as at 1/20 - Table 4).

7.7 (Cont'd.)

and 100-fold lower with a mixture of α VP7.2 and α VP10 than with α VP7.2 alone (table 9). α VP7.1 did not affect the neutralization caused by α VP7.2. (Note that with mixtures of antisera α VP7.2 was diluted 2-fold than when it was used alone (i.e. $1/40$ compared to $1/20$), but that neutralization with α VP7.2 was as efficient at $1/160$ as at $1/20$).

The results suggest that the other antisera with neutralizing activity against rotavirus, i.e. α VP6 and α VP10, have blocking effects on neutralization by α VP7.2 but that α VP7.1 has no such effect suggests that either the titres of α VP6 and α VP10 are much higher than that of α VP7.1 or that α VP6 and α VP10 bound closer to the binding site of α VP7.2 than did α VP7.1 or that they do not compete with α VP7.2 for binding but that their biological effects interfere with the specific neutralizing activity of α VP7.2. This experiment was repeated once only, which confirmed the blocking effects of α VP6 and α VP10.

7.8 Determination of the amount of α VP7.1 and α VP7.2 binding to virus particles.

The fact that α VP7.2 neutralized rotavirus and α VP7.1 had no effect on its infectivity when both antisera precipitated the same polypeptides may simply have meant that α VP7.1 was directed against determinants on VP7.1 and VP7.2 which were not exposed on the surface of the virus particle. This was investigated by estimating the relative amount of each

7.8 (Cont'd.)

immunoglobulin that bound to virus particles. Double shelled virus particles were fixed with formalin to prevent dissociation, purified by CsCl gradient centrifugation and mixed with ^{125}I -labelled monospecific immunoglobulins (which had both equal mass and equal incorporated c.p.m.) for 18h at 4°C. The virus with attached antibody was pelleted through 45% sucrose and the relative amount of each antibody bound to the virus particles was then quantitated. Approximately 2.8 times more $\alpha\text{VP7.1}$ bound to virus than $\alpha\text{VP7.2}$ (table 10). Since binding was inversely related to neutralizing activity, we conclude that there are more antigenic determinants on the rotavirus surface which react with $\alpha\text{VP7.1}$ than with $\alpha\text{VP7.2}$. Approximately 6 times more $\alpha\text{VP7.2}$ and 15 times more $\alpha\text{VP7.1}$ bound to complete particles than did αVP6 . VP6 is the major rotavirus polypeptide and is 3.3 times more abundant than VP7.1 and VP7.2 combined (see chapter 4). It also elicited an antibody response as quickly as VP7.1 and the immunofluorescent titre of αVP6 was as high as that of $\alpha\text{VP7.1}$ and $\alpha\text{VP7.2}$. Therefore, we conclude that only a little VP6 is exposed on the outer capsid surface of rotavirus or that our αVP6 is directed mostly against inner components of VP6. Neutralization by αVP6 must be mediated via this exposed portion of VP6 since single shelled particles (where VP6 is located) are not infectious.

TABLE 10

Binding of purified ^{125}I -immunoglobulins to rotavirus particles

Immunoglobulin	cpm bound to virus	
	uncorrected	less. non-specific binding (of preimmune Ig)
PRE-IMMUNE	946	-
αVP6	1,841	895
$\alpha\text{VP7.1}$	16,913	15,067
$\alpha\text{VP7.2}$	6,317	5,361

50 μg ^{125}I -Ig (1.5×10^6 cpm in each case) was mixed with about 5 μg formalin-fixed purified virus in a final volume of 1 ml PBS. After 18h at 4°C with stirring, the virus-antibody complexes were separated from free ^{125}I -Ig by centrifugation at $100,000 \times g$ through 45% sucrose for 90 min.

7.9 Reaction of monospecific antisera with calf rotavirus as determined by immune electron microscopy (IEM).

The immune electron microscopy experiments were done in collaboration with Dr. T. Flewett. Most samples were examined 'blind' by Dr. Flewett to avoid the possibility of bias in the detection of immune aggregates. Aggregated viruses were distinguished from naturally clumped viruses. Table II summarises the results of electron microscopic examinations of the reactions of all the monospecific antisera with three different virus samples viz. (1) faecal material from a gnotobiotic calf infected with calf rotavirus (see chapter 2.4), which had been diluted in PBS, stirred and centrifuged at $5,000 \times g$ for 20 min to clarify the suspension. This sample had been stored as faecal material for 2 years prior to use and the virus particles were mostly single shelled, (2) the same strain of calf rotavirus purified from tissue culture. This sample had fewer particles but a much higher proportion of double shelled particles and (3) virus from the faecal material which was treated with EDTA to determine whether EDTA treated virus and naturally occurring single shelled particles had the same antigenic composition.

Virus samples 1 and 2 gave the same results in reactions with the monospecific antisera. Calf anti-rotavirus serum (G203) aggregated both double shelled and single shelled particles. Interestingly, these aggregates were always of

Table 11 Immune electron microscopy: aggregation of calf rotavirus by monospecific antisera.

Serum	Faecal rotavirus ⁺ (part purified)		Faecal rotavirus + EDTA	Tissue culture ⁺⁺ virus(purified)	
	Aggregated	Type	Aggregated	Aggregated	Type
C1 serum	++	single shelled ⁺ double shelled	ND	-	
αVP1	ND		ND	-	
αVP2	-		-	-	
αVP3	ND		ND	-	
αVP4.2	ND		ND	-	
αVP6	-		-	-	
αVP7.1	* ++	double shelled	-	++	double shelled
αVP7.2	+	double shelled	-	++	double shelled
αVP10	+++	single shelled	++	++	single shelled
None	-	-	ND	-	
Preimmune	-	-	-	-	

* Faecal rotavirus was of the tissue culture adapted strain infected into a gnotobiotic calf. The virus was partly purified by centrifugation at 5,000 x g for 20 min followed by centrifugation of the supernatant through 45% sucrose at 100,000 x g for 90 min.

++ Tissue culture virus was grown in and purified from roller bottles of BSC-1 cells as previously described.

- = no aggregates seen

*+ = aggregates seen (up to 4)

++ = large aggregates seen (< 20).

+++ = aggregates (> 20).

7.9 (Cont'd.)

either single shelled or of double shelled particles rather than being mixed aggregates (fig.3). α VP7.1 and α VP7.2 both aggregated double shelled but not single shelled particles (fig.3). The neutralizing α VP7.2 was less effective than α VP7.1 at producing aggregates with the faecal sample, probably due to the low proportion of double shelled particles in this sample. This is also consistent with the greater amount of α VP7.1 that bound to virus (table 10), indicating that there are more antigenic determinants on the virus available to react with α VP7.1 than with α VP7.2, or that α VP7.1 contains more specific antibody to the virus surface.

α VP10 aggregated single shelled particles but not double shelled particles, although occasionally some double shelled particles were seen at the edges of the clumps (see fig.3). this presents a paradox since VP10 is a minor polypeptide present in double shelled particles but not in single shelled particles formed by EDTA treatment (see fig.1, chapter 6). One explanation could be that VP10 was still attached to naturally occurring single shelled particles. In reovirus, naturally occurring subviral particles in infected cells contain a fragment of the μ lc protein, but cores prepared by chymotrypsin treatment of reovirus are identical to subviral particles except they lack this fragment of μ lc protein (Joklik, 1974). To test this possibility, we prepared single shelled rotavirus by treating the

7.9 (Cont'd.)

faecal sample with EDTA, but we found that these single shelled particles were still aggregated by α VP10 (fig.3).

Surprisingly, neither α VP2 nor α VP6 aggregated single shelled or double shelled particles in any of the virus samples, although VP2 and VP6 between them comprise almost all of the viral inner shell protein (Novo and Esparza, 1981). Presumably these polypeptides do not express the appropriate antigenic determinants on the particle surface for aggregation even though they do induce antibody which reacts under denaturing conditions (IP and IF) and VP6 reacts to some extent in the neutralization reaction. α VP1, α VP3*, α 4* and α VP4.2, all of which only reacted weakly by immunofluorescence and did not react (except α VP3*, α 4*) by immune precipitation, failed to aggregate either double shelled or single shelled particles. However, as the titre of these antisera was obviously very low, we cannot make any conclusion about the status of these polypeptides in rotavirus particles.

The viral aggregates illustrated in fig.3 provide examples of those seen in the EM. Definite visualisation of antibody on the virus surface was hampered by less than optimal resolution due to extraneous protein and also by low concentrations of specific antibody in the monospecific antisera. However, in some cases (fig.3 b,e) antibody could possibly be detected, and in many others the virus particles

Fig.3 Immune electron microscopy using monospecific antisera.

(a) Naturally occurring virus particles before addition of antiserum, (b) predominantly double shelled particles aggregated and partly obscured by VP7.1, (c) double shelled particles aggregated by VP7.2, (d) single shelled particles from faecal material aggregated by VP10, (e) single shelled particles from virus purified from tissue culture aggregated by VP10 (note double shelled particles at edge), (f) double shelled particles aggregated by C1 antiserum, (g) single shelled particles aggregated by C1 antiserum.

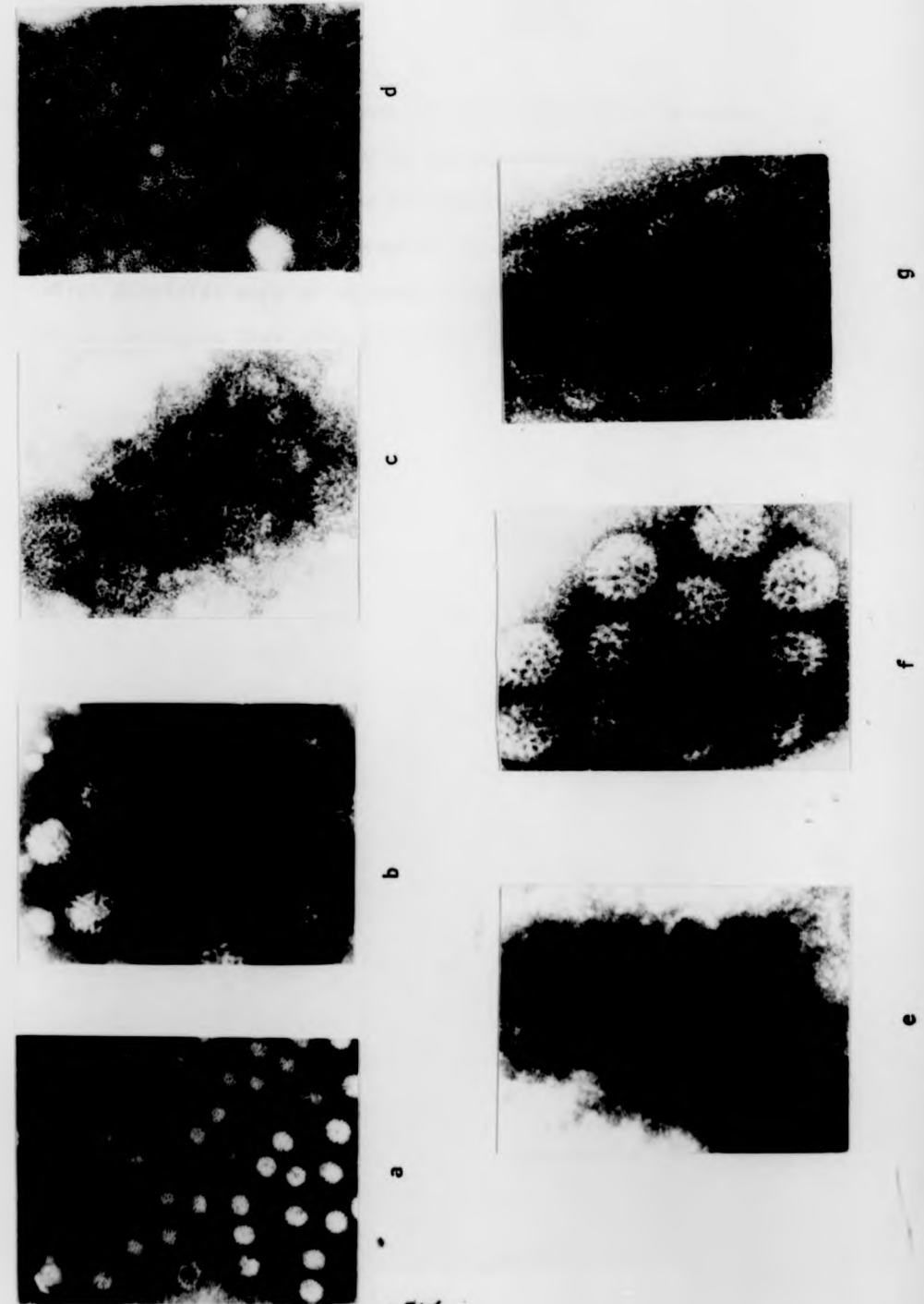
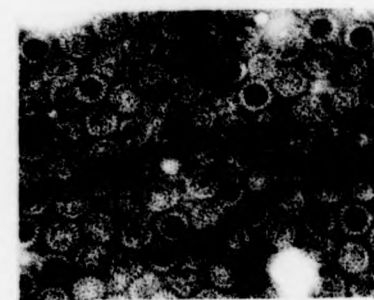
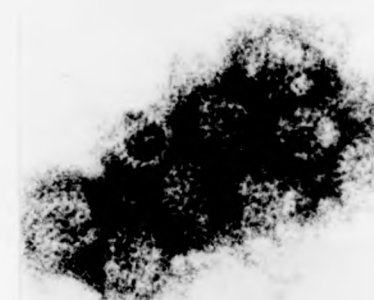


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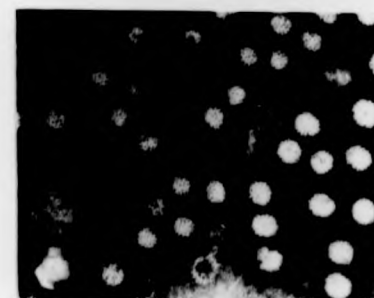
a



b



c



d



e



f



g

7.9 (Cont'd.)

had somewhat 'fuzzy' outlines (figs. 3 e,g) which is often a sign of antibody attached to the particles. Despite the lack of obvious antibody in the aggregates, these clumps were large and definite enough to be unmistakable and the virus particles were at an even distance from one another, which indicates that they are bound together by antibody.

CHAPTER 7

DISCUSSION

The properties of our monospecific antisera are summarised in table 12. The immune precipitation and immunofluorescence, and cross-reactions of the monospecific antisera with porcine rotavirus using these methods were described in chapter 6. All the other work has been described in this chapter. In particular, the location of antigens within the virus particles, and assignment of some of their biological functions were successfully determined by use of the monospecific antisera in reactions with virus particles. In contrast to work described in the previous chapter where immunofluorescence gives results on antigens which are possibly denatured due to fixation, and immune precipitation is done in highly denaturing conditions, IEM and neutralization assays use native antigen in the form of mature particles. However, some caution was necessary in interpretation of the results as the antisera were prepared with highly denatured (although possibly partly renatured) polypeptides, so these antisera may recognise determinants not normally seen in nature, and fail to recognise some important native determinants. However, the fact that monospecific antisera prepared in this way have been useful in characterising other viruses (as mentioned in the introduction to chapter 6) confirmed our opinion that the reactions observed between these antisera and native virus particles were genuine.

Table 12

Summary of the properties of the monospecific antisera.

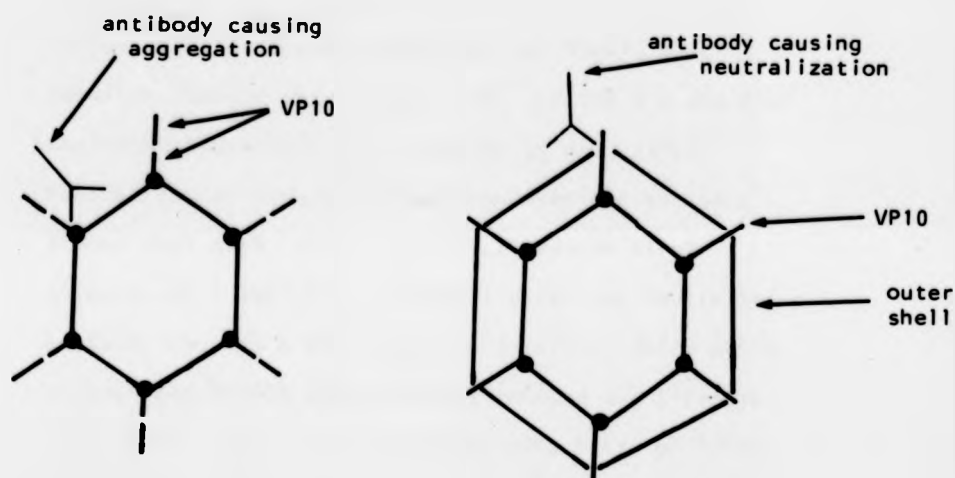
Serum against	Immuno- precipitation	Cytoplasmic Immuno- fluorescence	% Neutral- ization	immune	EM
				Ss	aggreg. Ds
VP1	-	±	0%		ND
VP2	VP2	+	0%	-	-
VP3*,4*	VP2	±	0%		ND
VP4.2	-	+	0%		ND
VP6	VP6	+	50%	-	-
VP7.1	VP7.1, VP7.2	+	0%	-	+
VP7.2	VP7.1, VP7.2	+	99%	-	trace
VP10	-	+	90% (variable)	++	-

1. Agglutination by monospecific antisera.

Agglutination of double shelled or single shelled particles by IEM with monospecific antisera identified which polypeptides were on the surface of either of these particle types. VP7.1 and VP7.2 were thus identified as proteins of the outer shell only, as expected. In contrast, α VP10 gave surprising results as it did not aggregate double shelled particles but only single shelled particles. Even single shelled particles treated with EDTA were aggregated by α VP10, although these particles appear to contain no VP10. This result has led us to believe that at least some of VP10 is attached to the inner shell. Several other groups have also identified VP10 as an outer shell protein (Bastardo et al., 1981; McCrae and Faulkner-Valle, 1981; Espejo et al., 1981; Sabara et al., 1982). We propose that VP10 is a component of both protein shells (i.e. attached to the inner shell but extruding through the outer shell) and that a reduced amount or part of VP10 remains attached to the inner shell in single shelled particles. As VP10 is already a minor polypeptide, it is conceivable that this reduced quantity would not be detected in single shelled particles by PAGE. Most of the antibody in our α VP10 must be directed against internal VP10 sites, with a small amount of antibody against external sites (since α VP10 neutralises

infectivity). As double shelled particles were occasionally seen on the edges of some clumps (fig.3), a low antibody titre to external sites on VP10 is quite plausible. Our proposal is outlined diagrammatically in fig.4. The lack of aggregation of particles by α VP2 and α VP6 was surprising because a) single shelled particles are almost completely composed of VP2 and VP6 (Novo and Esparza, 1981) and b) both of these antisera had high titres by immunofluorescence. Furthermore, Bastardo et al. (1981) reported that their α VP6, prepared in the same way, aggregated single shelled and double shelled particles. Thus it would appear that our α VP2 and α VP6 react with antigenic determinants of VP2 and VP6 which are not exposed on the outer surface of either of these particle types. However, the fact that α VP6 has some neutralizing activity must mean that some of it reacts with determinants of VP6 which are on the surface of double shelled particles, but the concentration may be too low to aggregate particles. The monospecificity of the α VP6 prepared by Bastardo et al. (1981) was not determined so the aggregation that they see may be due either to antibody against an antigenic site on VP6 that our antiserum preparation lacks or due to antibody against other proteins.

Fig.4 Diagrammatic representation of VP10 in virus particles.



a) Single shelled particles.
The breaks in VP10 indicate that VP10 may be cleaved upon loss of the virus outer shell.

b) Double shelled particles.

2. Neutralization

Neutralization of virus infectivity by antibody is of great importance and we wanted to determine which of our monospecific antisera neutralized rotavirus infectivity and thus identify the antigen(s) responsible for eliciting ^{neutralizing} antibody. In most viruses, this function can be mainly attributed to one antigen - e.g. HA of influenza virus (Laver and Kilbourne, 1966), $\sigma 1$ of reovirus (Weiner and Fields, 1978) and the G protein of vesicular stomatitis virus (Kelley *et al.*, 1972). Neutralization assays with our monospecific antisera showed that α VP6, α VP10 and α VP7.2 reduced viral infectivity. α VP7.2 was the most effective neutralizing antibody causing a 99% reduction in titre, while α VP10 neutralized 80-90% and α VP6 only reduced the titre by 50%. Also, α VP7.2 had a neutralizing titre of $1/640$, α VP10 had a titre of $1/160$ and the titre of α VP6 was $1/20$. In reovirus, where the neutralization-specific antigen, as defined by use of reassortant viruses, is $\sigma 1$, monoclonal antibody against $\sigma 3$ and $\lambda 2$ also neutralize (Hayes *et al.*, 1981). Use of rotavirus recombinants have shown type-specific neutralization to be a product of gene segment 8 or 9 (Kalica *et al.*, 1981) and the only structural proteins coded by these genes are VP7.1 and VP7.2 (McCrae and McCorquodale, 1982). This confirms

our conclusion that α VP7.2 is our major neutralizing antibody. The authenticity of neutralization by α VP6 was too low to investigate, but neutralization by α VP10 (or α VP7.2) was not reversed by sonication treatment of neutralized virus indicating that it was not caused by aggregation of particles and so was genuine neutralization. Aggregation of complete virus by α VP10 was not seen by IEM either. Both α VP6 and α VP10 had strong activity in other ways - α VP6 had a high immunofluorescent titre and α VP10 strongly aggregated single shelled particles and also had a strong immunofluorescent reaction.

The most important neutralization-specific antigen, VP7.2, is an interesting antigen as it is only one of a group of related rotavirus polypeptides and appears to be formed by extensive modification steps, unlike its reovirus counterpart σ 1, which is a primary gene product. In rotavirus, the primary gene product vpr7 first loses a signal sequence (Erickson *et al.*, 1983) and is then glycosylated forming VP7, which is cleaved to VP7.1 (McCrae and Faulkner-Valle, 1981). Although the relationship between VP7.1 and VP7.2 has been confirmed by peptide mapping (Espejo *et al.*, 1981), which showed VP7.1 to have all the peptides of VP7.2 plus at least one more and by our immune precipitation with monospecific antisera (chapter 6), the mechanism and kinetics of its formation has not been elucidated for two reasons -

it is masked by VP8 and VP9 in infected cells and it is present in small quantities in the cells and we have only been able to detect it by immune precipitation with α VP7.1 and α VP7.2. The discussion of chapter 4 outlines possible methods of formation of VP7.2.

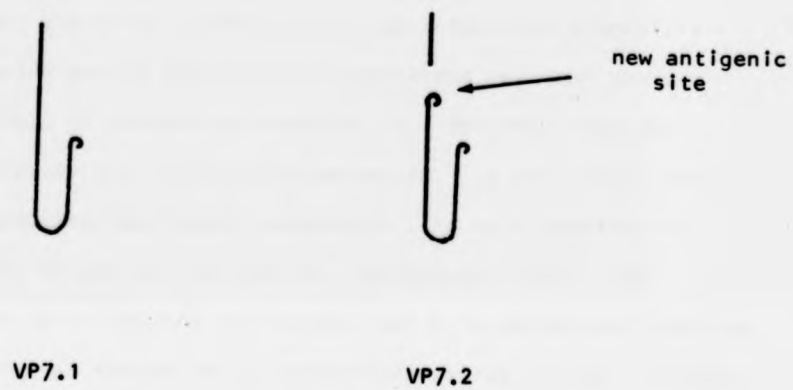
The fact that antiserum against VP7.1 precipitated the same polypeptides as α VP7.2 at least as efficiently and yet did not neutralize rotavirus infectivity does not conform to the theory that these two proteins are natural variants of each other present on different particles (see discussion of chapter 4, p. 216), nor does the fact that the only virus protein binding to cells in a binding assay comigrates with VP7.2 (or VP8) and not VP7.1 (chapter 6). From arguments presented in the discussion of chapter 4 and the fact that Estes et al. have monospecific antiserum against VP7.1 (Estes et al., 1982; Erickson et al., 1982; 1983; Petrie et al., 1982) and have not reported any neutralization with it, leads us to believe that either these viruses with variation in VP7 produce both VP7.1 and VP7.2, or that Estes et al. have some natural mutants deficient in processing of VP7. One of their isolates is definitely in this category as it does not glycosylate vpr7, yet is still infectious (Estes et al., 1982; Erickson et al., 1983).

Although our α VP7.1 may simply lack antibody to a neutralization-specific determinant present on both VP7.1 and VP7.2, it is more likely that VP7.2 has either acquired new antigenic determinants as well as losing those presumably cleaved from VP7.1, or that the cleavage of VP7.1 to VP7.2 has exposed new antigenic determinants that are important in neutralization (see fig.5).

3. Effect of complement on neutralization

The neutralization of infectivity by both α VP7.2 and α VP10 appeared to be partly heat labile - neutralization with α VP7.2 was reduced from 99% to 80% while that with α VP10 was reduced from 90% to 60% by heating at 56°C for 30 min. Purified immunoglobulin from α VP7.2 neutralized 93% infectivity and this was not significantly reduced on heating, indicating that the immunoglobulin itself was not heat labile. Addition of commercial guinea pig serum as a source of complement to heat treated α VP7.2 completely restored its neutralizing activity indicating that the neutralization was potentiated by complement. Complement (C') potentiation of virus neutralization has been frequently reported (Daniels, 1975), and can occur either by a lytic or non-lytic mechanism (Cooper, 1979). As rotaviruses do not have an envelope, lytic effects mediated by the phospholipase activity of C'7, C'8 and C'9 do not occur, and non-lytic mechanisms of virus neutralization occur only through the early components of the C' pathway - C'1, C'4,

Fig.5 Diagrammatic representation of the possible structure of VP7.1 and VP7.2.



C'2 and C'3 (Cooper, 1979). In cases where a mechanism for non-lytic potentiation of neutralization has been found, it has been either by envelopment of the virus particle or the active site by complement proteins or by aggregation of virus particles. Herpes simplex virus type 1 was neutralized by antibody and complement components C'1 and C'4, which coat the virus surface and avian infectious bronchitis virus neutralized by antibody and complement was seen to have a large halo of protein surrounding it. Polyoma virus was neutralized by the alternative mechanism - it was crosslinked by antibody and complement components C'1q or a combination of C1, C4, C3 and C2 (for review, see Cooper, 1979). Our rotavirus particles are not neutralized by a mechanism involving aggregation as sonication of neutralized virus did not increase its infectivity. Complement may coat the virus surface and thus potentiate neutralization. Calf rotavirus convalescent calf serum (G203) was heat stable, but the complement in G203 may have been inactivated due to long storage.

4. Synergistic effects of the antisera.

Despite the fact that three of our monospecific antisera have neutralizing activity against calf rotavirus, we found no positive synergistic effects by mixing the antisera, but

rather negative synergism between them. Therefore, unlike the situation with adenovirus, our antibodies raised against less or non-critical sites on rotavirus do not enhance the neutralization found with α VP7.2. α VP10, and α VP6 to a lesser extent, appeared to block neutralization caused by α VP7.2, possibly by steric hinderance of antibody attachment or because these antibodies prevent conformational changes induced by α VP7.2, which are needed for neutralization, from taking place. Blocking effects of this nature would obviously be a very important adaptation of a virus for evading the severe effects of immune responses in vivo, and enabling the virus to persist in the host. Lymphocytic^{cytic} chorio meningitis (LCM) virus, which causes persistent infection, is known to induce blocking antibodies (Mims, 1977).

5. Comparison with other work.

The identification of the rotavirus neutralization-specific antigen is obviously of major importance for serological analysis and for vaccine production. A very similar study to ours was done by Bastardo et al. (1981) with "monospecific" antisera prepared in rabbits against SA-11 rotavirus polypeptides. This group did not resolve VP7.1 and VP7.2. Their p26 and gp25 presumably correspond to our VP10 and VP10c (Dyall-Smith and Holmes, 1981) and these were both found in virus particles and injected separately. Neutralization experiments showed that three

of their antisera neutralized rotavirus infectivity - these were α VP7.1/7.2, α VP10 and α VP6 and of these only α VP7.1/7.2 had HI activity and none were type-specific. Since the specificity of their antisera was not checked in any way, there is no evidence that their sera were indeed monospecific, but their results agree generally with ours. They did not specify if their antisera were heat-treated before neutralization assays. No synergism was noted with their antisera when all were used together. Interestingly, antisera raised against non-reduced proteins were much more active than those against reduced polypeptides. The polypeptides we used for injection were reduced - this could have had an effect on the neutralizing titres of the antisera. Kaluza and Pauli (1980) studied the effect of 2-mercaptoethanol on Semliki Forest virus glycoproteins and found that the reductive cleavage of disulphide bridges by this agent changed the tertiary structure of the glycoproteins and resulted in the loss of haemagglutinating activity and infectivity of the virus. Our antiserum raised against denatured and reduced VP7.2 had neutralizing activity but it would be interesting to see if this was increased by using unreduced polypeptides as immunogen.

Recently monospecific antiserum has also been raised against VP7.1/7.2 by Matsuno and Inouye (1983). The antigen was prepared by milder denaturation using urea and fractionation by isoelectric focussing in glycerol gradients.

These antisera neutralized infectivity and one of the two preparations were type-specific. Greenberg et al. (1983a,b) have monoclonal antibodies which precipitate VP3, VP6 and VP7 from infected cells. α VP3 and α VP7 both neutralized infectivity and only α VP3 was type-specific so they have identified a fourth antigen involved in neutralization. All the α VP3 antisera had type-specific HI activity. Some monoclonal antibodies against VP7 had neutralizing and HI activity and some had no HI activity. As VP3 is the haemagglutinin in reassortment experiments (Kalica et al., 1983), the authors suggested that α VP7 could inhibit haemagglutination by steric hinderance of VP3.

The genes coding for type-specific neutralizing antigens were determined by reassortment between temperature-sensitive calf rotavirus and human rotavirus (Kalica et al., 1981) and only gene segment 9 (which is possibly segment 8 in calf rotavirus) was shown to determine the type-specific neutralization-specific antigen. Gene 8 of calf rotavirus is thought to code for the precursor vpr7. The gene coding for VP6 determined the subgroup antigen identified by ELISA even though VP6 is an inner shell polypeptide. Our α VP6 also neutralizes infectivity. The reassortment experiments did not assign a serological role encoded by the gene for VP10 (segment 10), although this gene is the most variable in mobility (Dyall-Smith and Holmes, 1981b) and was previously thought to be related to type-specificity. This reassortment data confirm our

observations that VP7.2 is the most important antigen involved in neutralization. As mentioned above, haem-agglutination has been assigned to gene segment 4 - i.e. VP3 - (Kalica et al., 1983) - by reassortment.

6. Comparison to reovirus

More than one antigen associated with neutralization has also been observed in reovirus in experiments using monoclonal antibodies (Hayes et al., 1981), although reassortment experiments had only shown the gene for $\sigma 1$ to encode a type-specific, neutralization-specific antigen (Weiner and Fields, 1977). Monoclonal antibodies against $\sigma 1$, the major outer shell polypeptide $\sigma 3$ and the core protein $\lambda 2$ all neutralized infectivity (Hayes et al., 1981), although $\sigma 1$ was the most type-specific. The authors concluded that the inner shell polypeptide $\lambda 2$ protrudes through the outer shell onto the surface of the virus. All antisera which neutralized infectivity also aggregated reovirus particles. $\lambda 2$ is analogous to the rotavirus polypeptide VP10 which was thought to be a minor outer shell polypeptide, as it appears to be absent in single shelled particles analysed by PAGE, but single shelled particles and EDTA treated virus are agglutinated by α VP10. Thus it is likely that VP10 is present in both inner and outer shells and it could be partially removed or cleaved by EDTA or other mechanisms for removing the outer shell. Alternatively, this antiserum may cross-react with another rotavirus polypeptide involved in neutralization,

as we have no independent criterion to decide if α VP10 is monospecific, as it did not precipitate any polypeptides. However, this makes it unlikely to have antibody to proteins which we know are precipitated. The antisera described by Bastardo et al. (1981) against VP10 and VP10c aggregated complete particles, but only antisera against non-reduced polypeptides did so and α VP10 was more effective than antiserum against VP10c, our structural protein. Unfortunately, Bastardo et al. (1981) did not test for aggregation of single shelled particles.

VP7.2 is analagous to σ 1, the main neutralization-specific antigen of reovirus, as they are both minor outer shell components and among the smaller of the virus polypeptides. However, σ 1 is a primary gene product and is not glycosylated (McDowell et al., 1972). σ 1 is also responsible for binding to cell receptors and for HA. Evidence has been presented in this thesis (section 6.6) that a polypeptide migrating in the region of VP7.2 binds to cells, and Matsuno and Inouye (1983) have also found that α VP7.1/7.2 inhibits virus from binding to cells. Bastardo et al. (1981) have shown that it is involved in haemagglutination although Greenberg et al. (1983b) found α VP3/4.2 is also involved. Reovirus σ 1 has also been implicated in specifying tissue tropism virulence and the extent of association with microtubules (Weiner et al., 1977; Babiss et al., 1979) as

well as being responsible for development of delayed hypersensitivity and the generation of cytotoxic T lymphocytes and suppressor T cells (Weiner et al., 1980; Finberg et al., 1979; Fontana and Weiner (1980). Similar experiments would be difficult to do with rotaviruses, as the genetic approach employed in the reovirus experiments could not directly implicate rotavirus VP7.2 or in fact any rotavirus outer shell polypeptide, as none are primary gene products.

Further studies on the structure of VP7.1/VP7.2 and VP10/VP10c are also hampered by processing involved in their production, and techniques for isolation of these proteins in their natural state have not been established. The cloning of genome segments encoding for these proteins is under way (McCrae and McCorquodale, 1982). It has not yet been established whether the segments that have been cloned are full length inserts. It also remains to be seen if they are expressed in E.coli, or if they become properly and completely modified. The cloning will provide primary sequence data, and the possibility that the cloned gene products could serve as a potential vaccine will certainly be investigated.

Concluding remarks

The identification of rotavirus proteins which are important in eliciting neutralization-specific antibodies in the previous chapter was preceded by extensive characterisation of rotavirus polypeptides by biochemical and serological methods, and some important conclusions were made in these earlier chapters. The preliminary characterisation of rotavirus mRNA also yielded some useful information on the mechanism of rotavirus transcription and translation.

Characterisation of the rotavirus primary gene products made by in vitro transcription and by in vitro translation provided the basis for investigating the extensive post-translational processing involved in the production of structural proteins. The enzymes necessary for transcription and for post-transcriptional modification of rotavirus RNA were seen to be contained in the virus particle since fully functional mRNA's could be produced in vitro. The mRNA is transcribed with great efficiency and resembles reovirus mRNA as it is segmented, and is capped and methylated at the 5' ends but is not 3' polyadenylated. The mRNA species were also translated very efficiently in vitro - most were completely translated into full length proteins and only one possible product of premature termination or a second reading frame was identified. The higher molecular weight

mRNA's were translated less efficiently in vitro than in vivo.

When primary gene products were compared to virus-specified proteins from infected cells and to rotavirus structural proteins, it was seen that 8 of the 11 structural proteins are the products of extensive post-translational modification including glycosylation, natural cleavage and trypsin-induced cleavage. This result was unexpected since all but one of the reovirus structural proteins are primary gene products. The rotavirus proteins, VP7.1 and VP7.2, are formed by glycosylation of vpr7 to VP7 and cleavage of VP7, and VP10c is formed by glycosylation of VP12 to VP10 and subsequent trimming of VP10. These steps take place during virus replication in infected cells. The virus particles produced in infected cells are not infectious unless an external source of trypsin is present. The trypsin cleaves VP3 to VP4.2 and VP4.3 in the virus particle and this step enhances the virus infectivity. Some of VP2 is also cleaved by trypsin to produce VP3*,4* and VP5*.

Most of the proteins which become modified are exposed on the outer surface of the virus particle and have important serological functions. The inner shell proteins, which are suspected to have the enzyme activities such as transcription and capping methylation of the transcripts, are mostly primary gene products and are group-specific (or subgroup-specific) antigens. In infected cells, VP3, VP4.2 and VP7/7.1, which are destined for the outer shell, appear to have type-specific properties in cross-immune precipitation.

Using monospecific antisera raised against each of the structural polypeptides, the relationships between polypeptides were confirmed - for example VP7, VP7.1, VP7.2 and vpr7 were all precipitated by α VP7.1 or α VP7.2, and VP2 was precipitated by α VP3*,4*. Most of the rotavirus polypeptides were seen to be confined to the perinuclear cytoplasm in infected cells by indirect immunofluorescent staining, although α VP6 showed up some fluorescent spots in the nucleus. None of the rotavirus polypeptides appeared to be associated with the plasma membrane at any stage.

The monospecific antisera also provided some information about the location of the rotavirus polypeptides in the virus particle. In immunoelectron microscopy α VP7.1 and α VP7.2 agglutinated double shelled particles, suggesting that these two polypeptides are exposed on the outer surface of the virus particles. Binding of α VP7.1 and α VP7.2 to formalin fixed double shelled particles confirmed this. Some α VP6 also bound to double shelled particles although VP6 is said to be an inner shell protein. The fact that α VP6 can neutralize rotavirus infectivity also suggests that at least part of VP6 is exposed on the virus' outer shell surface. α VP10 agglutinated single shelled but not double shelled particles (although some double shelled particles were seen at the edges of the clumps). VP10 is said to be an outer shell protein and α VP10 neutralizes infectivity, so we conclude that part of VP10 is also found on the inner shell of the virus particle.

α VP7.2, α VP10 and α VP6 all neutralized rotavirus infectivity. α VP6 was inefficient as it only neutralized 50% of the virus infectivity, while α VP10 and α VP7.2 neutralized 80-90% and 99% of rotavirus infectivity respectively. Genetic experiments had shown that the gene coding for VP7.2 was responsible for type-specific neutralization (Kalica et al., 1981). Our experiments also differentiate between the neutralization-specific properties of VP7.2 and VP7.1 as α VP7.1 did not neutralize although it precipitated the same polypeptides as α VP7.2 and bound in greater quantity to virus particles than α VP7.2. The previous genetic experiments did not implicate VP10 in type-specific neutralization, although α VP10 clearly neutralizes rotavirus infectivity quite efficiently in our experiments. It seems, therefore, that neutralization is not a simple one protein/one antibody event, but that it can occur through antibody binding to several different proteins.

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